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# **The role of IL-4 receptor alpha in chronic allergic airway disease (AAD)**

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Thesis submitted to the University of Cape Town in fulfilment of the degree  
Doctor of Philosophy

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## **DECLARATION**

I, Jaisubash Jayakumar, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part thereof is being, has been, or is to be submitted for another degree in this or any other University.

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Jaisubash Jayakumar

February 2013

## **DEDICATION**

This thesis is dedicated to my late father Jayakumar Anandadoss, my beloved mother Krishnammal Jayakumar and my brother Jaianand Jayakumar.

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## List of Symbols and Abbreviations

Abbreviation or symbol	Description
-/-	Knockout
<	Less than
>	More than
<sup>0</sup> C	Degrees Celsius
γc	Common gamma chain
7aad	7-Aminoactinomycin D
AAM	Alternatively activated macrophage
AAD	Allergic Airway Disease
Ab	Antibody
AHR	Airway hyperresponsiveness
Alum	Aluminium hydroxide
AP	Alkaline phosphatase
APCs	Antigen presenting cells
APC-	Allophycocyanin-conjugated protein
Arg1	Arginase- 1
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar lavage (BAL)
BSA	Bovine serum albumin
CAM	Classically activated macrophage
CD	Cluster of differentiation
CXCL8	Chemokine ligand 8
CCL4	CC-Chemokine ligand 4
COPD	Chronic obstructive pulmonary disorder
Cre	Cre recombinase
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
E	Airway elastance
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immuno-sorbent assay
FACS	Fluorescence-Activated Cell Sorting
Fc	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
H&E	Haematoxylin & Eosin

H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HRP	Horse radish peroxidase
i.p	intra peritoneal
IFN- $\gamma$	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-4R $\alpha$	Interleukin-4 receptor alpha
IL-4R $\alpha^{-/-}$	IL-4R $\alpha$ deficient
IL-4R $\alpha^{-lox}$	Hemizygous for IL-4R $\alpha$ (considered wild type controls)
IL-13R $\alpha$	Interleukin-13 receptor alpha
IM	Interstitial macrophages
IMDM	Iscoe's Modified Dulbecco's Media
IRS	Insulin like receptor substitute
ISPF	Isonitrosopropiophenone
JAK	Janus Kinase
LysM	Lysozyme M (or abbrev. for LysM <sup>Cre</sup> IL-4R $\alpha^{-lox}$ mice)
LysM <sup>Cre</sup> IL-4R $\alpha^{-lox}$	Macrophage/neutrophil specific disrupted IL-4R $\alpha$
mAbs	Monoclonal antibodies
mRNA	Messenger RNA
MCh	acetyl-beta-metacholine
MHCI / II	Major histocompatibility complex Class I / II
MOX	Oxidised phospholipid-treated murine macrophages
MR	Mannose receptor
MSB	Martius scarlet blue
MUC	Mucin encoding gene
ND	Non detectable
NK	Natural Killer cells
NKT	Natural Killer T cell
NO	Nitric oxide
iNOS	Nitric oxide synthase 2 (or inducible NOS)
OD	Optical density
OVA	Ovalbumin
PAS	Periodic acid-schiff reagent
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Medium containing penicillin and streptomycin
PMA	Phorbol 12-myristate 13-acetate
PNP	P-Nitrophenyl phosphate
PPAR- $\gamma$	Peroxisome proliferator-activated receptor
qRT-PCR	Quantitative real-time PCR

R	Airway resistance
RAST	Radio allergosorbent testing
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean
SNPs	Single Nucleotide Polymorphisms
STAT	Signal transducer and activator of transcription
TAM	Tumour associated macrophages
TGF- $\beta$	Tumour growth factor beta
Th	T-helper (CD4 <sup>+</sup> ) cell
TNF	Tumour necrosis factor
UCT	University of Cape Town

# **ABSTRACT**

Asthma, a chronic inflammatory disease of the lungs affecting approximately 300 million people worldwide involves airway inflammation that is associated with airway remodelling. The IL-4 receptor alpha (IL-4R $\alpha$ ), which is the signal transducing receptor chain for IL-4 and IL-13, is known to play an important role in airway hyperreactivity (AHR) and lung pathology in asthma. Mouse models of AAD mimicking features of asthma such as airway hyperreactivity (AHR) and inflammation have demonstrated an important role for the IL-4R $\alpha$ , IL-4 and IL-13 in the pathology of this disease. However, the effects of IL-4 and IL-13 signaling through specific cell types in allergic asthma remain to be elucidated. The aim of our study is to investigate the role of IL-4/IL-13 target cells in allergic airway disease using comparative experimental cell type-specific IL-4R $\alpha$  deficient mouse models of chronic allergic airway disease.

IL-4R $\alpha$ <sup>-lox</sup> littermate control mice, IL-4R $\alpha$ <sup>-/-</sup> mice and mice with cell specific impairment of IL-4R $\alpha$  on CD4<sup>+</sup> T cells (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) or macrophages and neutrophils (LysM<sup>Cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) were sensitized subcutaneously with 25 micrograms of ovalbumin (OVA) adsorbed to alum at days 0, 7, 14 and 21 and subsequently challenged intranasally with 20 micrograms of OVA twice weekly for four weeks. Airway hyperreactivity (AHR) was measured by flexivent in response to increasing doses of methacholine and samples were taken for analysis.

Airway resistance and elastance were significantly increased in OVA-challenged IL-4R $\alpha$ <sup>-lox</sup> littermate control mice compared to PBS controls. Both global IL-4R $\alpha$ <sup>-/-</sup> and Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> OVA mice had significantly decreased airway resistance and elastance, reduced airway inflammation, mucus hypersecretion and airway eosinophilia compared to OVA IL-4R $\alpha$ <sup>-lox</sup> littermate controls and LysM<sup>Cre</sup>IL-4R $\alpha$ <sup>-lox</sup> OVA mice. Compared to IL-4R $\alpha$ <sup>-lox</sup> littermate control mice sensitized and challenged with PBS, OVA-challenged IL-4R $\alpha$ <sup>-lox</sup> littermate control mice had increased AHR after methacholine challenge. Allergic airway inflammation (airway infiltration, mucus production, Collagen deposition and airway remodelling) were reduced in Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice whereas it was maintained in LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice. Th2 type antibody, Th2 cytokines and Th17 responses were altered in Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice whereas those were unaltered in LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice.

Together, these results suggest that IL-4/IL-13-activated alternative macrophages are involved in AHR and IL-4-promoted Th2 cells are important in allergic inflammation and remodelling during OVA-induced chronic allergic airway disease.

# **CHAPTER 1**

## **General Introduction**

University of Cape Town



# 1 General Introduction

## 1.1 Overview of Allergy and Asthma

### 1.1.1 Allergy

Allergies are the most common chronic immunological disorders where an abnormal reaction of a person's immune system occurs in response to harmless substances in the environment. Allergic diseases include anaphylaxis, angioedema, asthma, food allergies, eczema, rhinitis and urticaria. There is a dramatic increase in the prevalence of allergic diseases globally with about 30-40% population being affected by it. The greatest burden of the rising trend of these diseases in the last few decades is found among children.<sup>1</sup> The two broad categories of risk factors for allergy include host and environmental factors.<sup>2</sup> The role of host heredity and genetic factors in the disposition to allergic diseases has been reported. However genetic factors in the host alone cannot provide insights into the recent increased incidence of allergic diseases even though polymorphisms in several candidate genes of allergic diseases have been reported.<sup>3</sup> Individuals with a known family history of allergies are more prone to develop allergies in their lifetime.

Allergy is a type I hypersensitivity reaction mediated by allergen specific immunological mechanisms causing excessive activation of mast cells and basophils by immunoglobulin E resulting in an inflammatory response which can range from uncomfortable to life threatening. Type I reactions, a class of humoral immune reactions occurs within minutes of contact with the allergen and due to its rapid onset are termed immediate hypersensitivity reactions. They are predominantly mast cell-mediated and are caused specifically by antibody of the IgE isotype which are capable of binding to Fc-receptors for IgE on mast cells found in tissue. Cross-linking of these membrane-bound IgE's by specific antigen results in mast cell degranulation releasing proallergic mediators such as histamine, leukotrienes and prostaglandins from mast cells and basophils. This eventually results in the myriad symptoms of allergy.<sup>4</sup>

The interplay between gene-gene, gene-environment, gene-gene-environment and gene-environment-environment interactions contributes to the risk of developing asthma thus emphasising that environmental factors also contribute to asthma in a genotype dependent

manner.<sup>5</sup> The hygiene hypothesis is supported by various epidemiological studies and explained the increased incidence of these diseases in developed countries thereby providing a vital framework in understanding the allergic diseases.<sup>6, 7</sup> Even though the reduction in the risk of developing allergies in children on exposure to certain infections in early childhood has been shown, the validity of hygiene hypothesis has been questioned.<sup>8-10</sup> The two possible explanations for this hypothesis are either early exposure to the allergen induces the development of a protective Th1 type immune response to the allergen, preventing a Th2 type immune response leaving the body more susceptible to developing Th2 induced disease.<sup>7, 11</sup> or the development of suppressive immunoregulatory responses to chronic or repeated infections.<sup>12</sup>

### 1.1.2 Allergens

Any antigenic substance responsible for eliciting an allergic response by stimulating a type-I hypersensitivity reaction through IgE responses is termed an allergen. Table 1.1 below lists some of the common allergens and their sources. Allergens can only be effective in causing an allergic reaction in an individual if there is previous sensitization to the allergen characterised by the induction of the IgE response directed against the allergen in the tissues and includes modulation of dendritic cells, B cells and T cells.<sup>13</sup>

**Table 1.1: Common allergens**

Source of allergen	Examples
Food	Eggs, fish, fruit, milk, nuts
House dust	Dander from house pets, mites
Mould	Food mould, plant mould
Plant Oils	Oak, poison ivy, sumac
Pollen from grasses, trees and weeds	Timothy grass, elm, ragweed
Venom from insects or animals	Bees, wasps, yellow jackets, fire ants, scorpion

Allergens are mostly proteins that are capable of reaching and stimulating immune cells capable of inducing an allergic response.<sup>14</sup> An allergic reaction is usually caused by any form of exposure to the dietary or environmental allergens via one of the following routes—ingestion (natural or artificial food products), inhalation through the air (pollen), direct contact (with the skin) or injection (drugs or insect bites).

Allergens are named using the systematic nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies. The system uses abbreviated Linnaean genus and species name of their source as follows: first three letters of the genus name, first letter of the species and an Arabic number to indicate the chronology of allergen purification. (E.g. Can f1 (Lipocalin) for domestic dog *Canis familiaris*).<sup>15, 16</sup>

### **1.1.3 Immunological mechanisms of allergy**

Both cellular and humoral immune responses are involved in allergies. Allergen specific IgE antibodies induce most allergic responses in humans. Any IgE-mediated reaction to common environmental allergens is called atopy and has got a strong genetic predisposition. In most humans, significant IgE responses are mounted against parasitic infections. In atopic individuals, non-parasitic antigens stimulate inappropriate IgE production, leading to type I hypersensitivity. Sensitivities vary widely from one person to another. A very broad range of substances can be allergens to sensitive individuals. IgG antibodies (e.g. drug induced anaphylaxis) or allergen specific lymphocytes (e.g. non-atopic asthma) can also induce allergic reactions.<sup>17, 18</sup>

#### **Sequence of events in allergies**

Immune responses to allergies are divided in-to two main phases:

- 1) Sensitization phase and
- 2) Reaction Phase

During the sensitization phase, an immune response to the allergen is established by the immune system on encounter with the allergen for the first time resulting in the induction of IgE antibody. IgE antibody binds to Fc receptors on the surface of mast cells (and basophils)

through its Fc portion. Dendritic cells (DCs) or antigen presenting cell (APCs) then present the allergen to the undifferentiated T-helper cells which then undergo differentiation and clonal expansion of allergen-specific T helper 2 (Th2) cells into allergen specific Th2 cells in the presence of IL-4 produced by various cells of the adaptive immune system.<sup>19</sup> B cells are then stimulated by allergen specific Th2 cells to produce allergen specific IgE or IgG1 antibodies. The establishment of an allergen specific T cell and B cell response to the allergen is critical for the further development of the allergic disease. These cells activate a memory response on second encounter with the allergen.

During the reaction phase, re-exposure to the allergen results in the binding of the allergen to mast-cell-bound IgE antibody, causing IgE cross-linking. The crosslinking of mast-cell and basophil cell-surface with the high-affinity receptor for IgE (FcεRI) bound IgE by allergens causes the release of large, basophilic granules into the tissues by the process of degranulation releasing a variety of inflammatory mediators : chemokines (such as CXC-chemokine ligand 8 (CXCL8), CC-chemokine ligand 4 (CCL4), cytokines (such as IL-4, IL-5 and IL-13), lipid mediators (such as leukotrienes, prostaglandins), proteases, and vasoactive amines ( histamine)<sup>20</sup> thereby increasing vascular permeability and smooth muscle cell contraction.<sup>21</sup> The expression of these cytokines recruits Th2 cells and eosinophils which play an essential role in the late phase of allergic reactions and subsequently chronic inflammation. Histamines, one of the chemoattractant for mast cells and eosinophils increases the vascular permeability enabling the influx of inflammatory cells at the site of the allergic reaction which in turn establish the chronic and late phase of AAD.<sup>22</sup> Eosinophils and basophils in circulation and mast cells in tissue are the key players in allergic reactions. Allergens bind to IgE on the surface of mast cells through histamine receptors releasing histamines stored in their granules causing injury to the surrounding tissue including blood vessels and nerve cells. Interaction of histamine with receptors on blood vessels causes increased leakiness, leading to the fluid collection, swelling and increased redness. IgE receptor also stimulates pain receptors, making tissue more sensitive and irritable.

#### **1.1.4 Diagnosis and treatment of allergy**

Allergy testing involves skin or blood tests to determine which allergen elicit an immune response. Skin tests are normally done due to their rapidness, reliability and cost effectiveness. In skin testing, a small amount of a suspected allergen is placed on or below

the skin to see if a reaction develops. The three types of skin tests are skin prick test, intradermal test and skin patch test. Blood tests utilize ELISA to look for allergen specific IgE antibodies in blood. Other laboratory testing methods include radioallergosorbent testing (RAST) or an immunoassay capture test (ImmunoCAP, UniCAP, or Pharmacia CAP).<sup>23</sup>

Common management for allergic conditions include avoidance of known allergens. However normally this is practically impossible. The treatments include use of variety of medications and also allergen-specific desensitization (immunotherapy). Medications such as anti-histamines specifically prevent allergic symptoms, steroids suppress immune responses, and decongestants reduce the symptoms of allergy. These medications are mostly administered orally. However, epinephrine which is used to treat anaphylactic reactions is injected. Immunotherapy uses injected allergens to desensitize the body's response.<sup>24</sup>

### **1.1.5 Asthma**

Asthma is a severe chronic inflammatory disease of the lungs. According to the WHO estimates, worldwide approximately 300 million people are affected by asthma, with 180 000 deaths resulting annually from severe asthma attacks.<sup>25</sup> Asthma poses a serious public health problem in all countries across the globe as shown in figure 1. Over 80% of asthma deaths occur in low and lower-middle income countries. Asthma has become the most common chronic disease among children and is one of the major causes of hospitalization among those younger than 15 years of age.<sup>26</sup> Triggers such as indoor allergens (house dust mites, pet hair or dander etc), outdoor allergens (pollens and mould), cold air, tobacco smoke, chemical irritants in the air or in the food, respiratory infections (common cold), extreme emotions, physical exercise, certain medication such as aspirin, non-steroid anti-inflammatory drugs, beta-blockers etc can cause asthma.

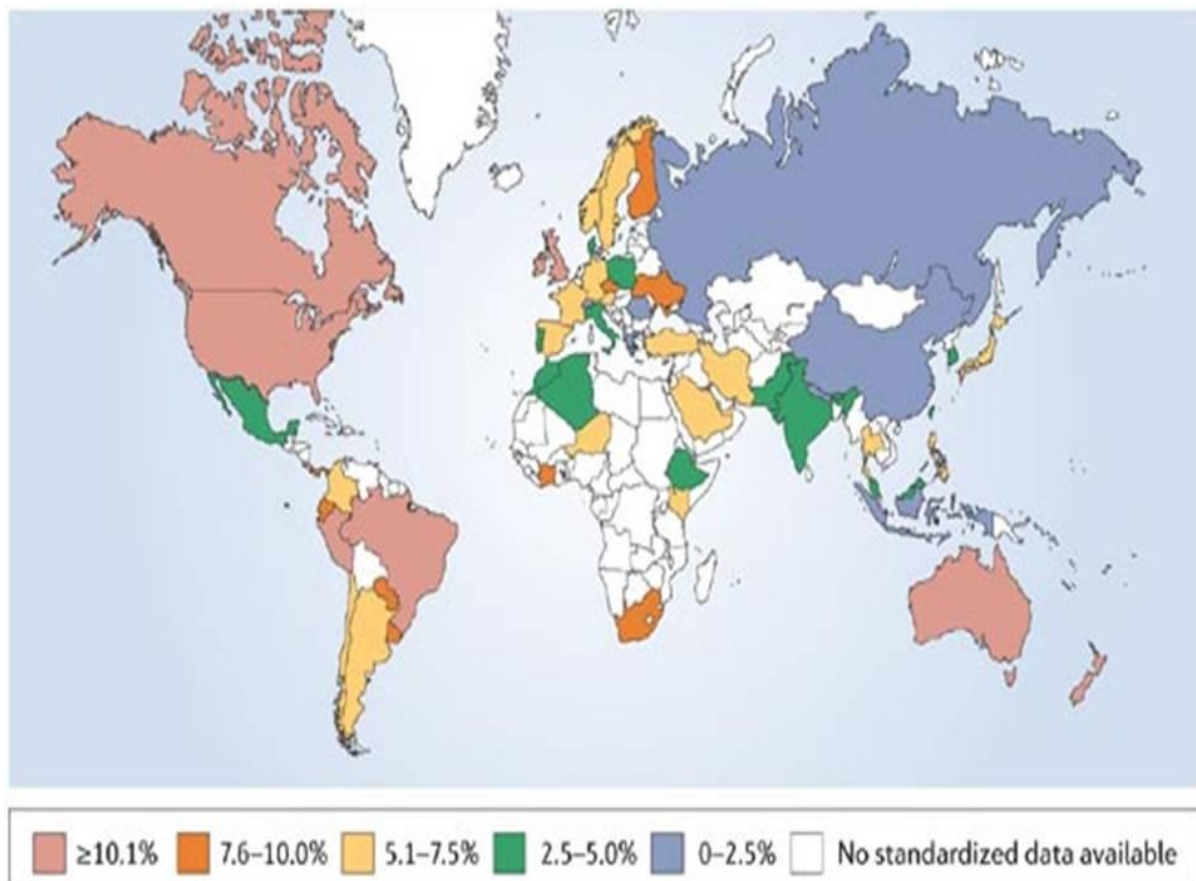
Asthma is characterised by recurrent episodes of symptoms such as coughing, wheezing, chest tightness, and shortness of breath, which vary in severity and frequency from person to person. In severe cases it can be fatal.<sup>27</sup> Even though the primary causes of asthma are not completely understood, genetic predisposition combined with environmental exposure to certain inhaled substances and particles were identified as the strongest risk factors for developing asthma.<sup>28</sup> The symptoms of asthma are caused by airway obstruction due to bronchoconstriction, mucus hypersecretion and inflammation of the airways that leads to airway wall thickening.<sup>29</sup> The majority of asthma is associated with allergy, and allergens can

trigger bronchoconstriction due to the release of inflammatory mediators from mast cells and basophils after IgE-crosslinking.<sup>30</sup>

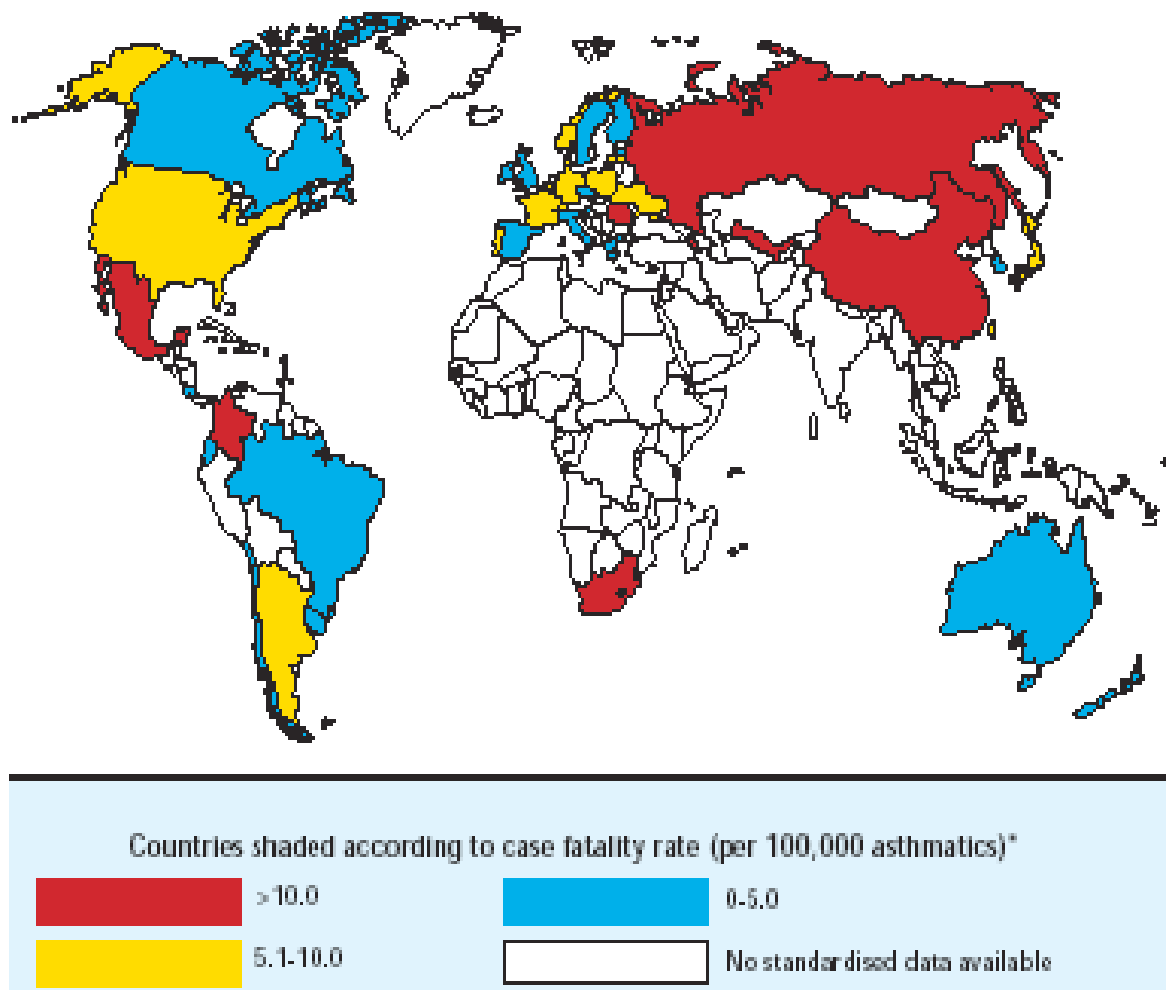
T helper 2 (Th2) responses occur in most asthma and mouse models of asthma. Increased numbers of alternatively activated macrophages and natural killer T cells have also been found in asthmatic airways<sup>31, 32</sup> and asthma exacerbations are associated with neutrophils.<sup>33</sup> There is also evidence to suggest that asthmatics have intrinsic differences in the epithelial cells of their airways that lead to impaired barrier function and enhanced signalling with underlying immune and mesenchymal cells.<sup>27, 34</sup> Differences in smooth muscle cells isolated from asthmatic patients including increased proliferation and increased production of connective tissue growth factor have been reported<sup>35</sup>. A number of factors may therefore contribute to the airway obstruction that is associated with acute asthma attacks.

Asthma cannot be cured. However, appropriate management of the disease using short-term and/or long-term medications can control and prevent the symptoms and exacerbations, thus enabling the patients a good quality of life. Avoidance of known triggers and exposure to risk factors is also important in controlling asthma. Current asthma therapy does not seem to be effective against certain types of asthma, particularly severe asthma as it uses chronic medication which has to be taken life long, involves certain side-effects and combat only the symptoms of the disease and not the disease itself. The most effective treatment for asthma is corticosteroid therapy which involves suppression of leukocytes, particularly Th2 cells.<sup>36, 37</sup> However, limited efficacy has been shown with agents such as cyclosporine that suppress activated T cells.<sup>38, 39</sup> Furthermore, the results of clinical trials that have tested monoclonal antibodies against individual Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13 have been disappointing overall.<sup>34</sup>

Asthma does not kill on the scale of chronic obstructive pulmonary disease (COPD) or other chronic diseases, but failure to use appropriate medications or to adhere to treatment can lead to death.<sup>40</sup> Also in developing countries, lack of access to hospitals and other health care facilities results in the increase of the mortality rate due to asthma as illustrated in figure 2.



**Figure 1. Global prevalence of asthma.** Map showing the variation in the prevalence of asthma. The percentage of asthmatic individuals differs in each country. Map from Graham Devereux 2006.<sup>41</sup>



**Figure 2. Global fatality rates of asthma.** Map showing the variation in the fatality rates of asthma across the globe. Map from Masoli M et al, 2004.<sup>42</sup>



## 1.2 Mouse models of Allergic Airway Disease (AAD)

Mouse models of allergic airway disease have been extremely useful in understanding the pathology of asthma as they share lot of similarities with human asthma.<sup>43</sup> In order to induce allergic reactions in mice, sensitization with ovalbumin (OVA) with the adjuvant is carried out first followed by the nasal administration of OVA without the adjuvant. These models are widely used to investigate the underlying immunological mechanisms of OVA- induced allergic airway inflammation.<sup>44-50</sup> However, besides OVA, allergens such as *Aspergillus* conidia,<sup>51, 52</sup> cockroach antigen<sup>53</sup>, dust mite antigen<sup>54</sup> and inhalant fish allergen<sup>55</sup> have also been used in other murine models of AAD.

Allergic airway disease (AAD) is a complex inflammatory disease involving many cell types, mediators and tissue responses. It involves a typical Th2 type immune response. Systemic immunization prior to airway exposure has been used in various murine models. Some of these models require systemic immunization of allergen prior to challenge and these allergens always need to be administrated together with an adjuvant (aluminium hydroxide (alum) or cholera toxin). The mice are systemically sensitised by repeated intra peritoneal (i.p) injection of allergen and adjuvant.

Inhalation of allergens in mice induces responses that are similar to human asthma: Production of Th2 cytokines, goblet cell hyperplasia, bronchoalveolar lavage and pulmonary neutrophil response, followed by eosinophil response, mast cell degranulation and IgE production, airway hyper responsiveness (AHR) to cholinergic stimuli that includes rapid and delayed development of increased airway resistance as shown in figure 3. Both human asthma and mouse models show memory responses as well.<sup>56</sup> However, studies in both animal and human have demonstrated the development of tolerance on repeated airway exposure to an antigen.<sup>49, 57-62</sup> indicating that instead of tolerance it leads to the disease development in asthma.

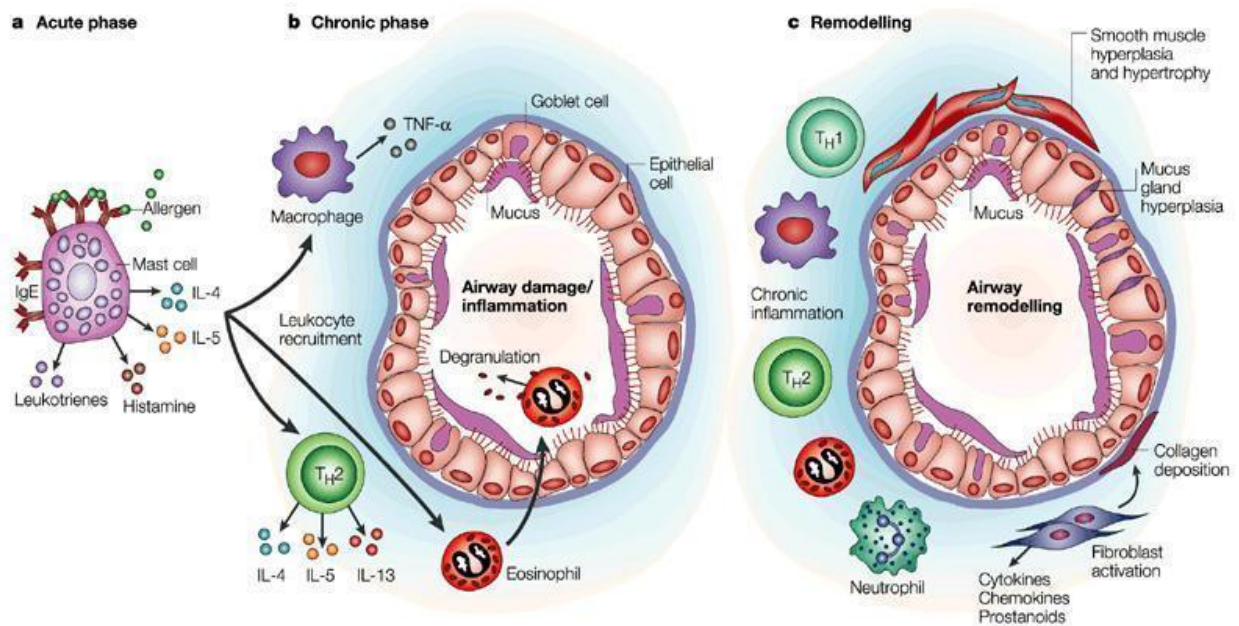
Chronic remodelling with smooth muscle hyperplasia and subepithelial fibrosis has been shown by some mouse models as shown in figure 3 (c).<sup>63</sup> Both human asthma and some mouse models are suppressed to some extent by leukotriene antagonists and leukotriene receptor antagonists, mast cell depletion, IgE antagonists, phosphodiesterase 4 antagonists, and corticosteroids.<sup>64</sup> It has also been reported that intraspecies genetic variability also

influences susceptibility to AAD in both mice and human subjects.<sup>65</sup> In humans, allergies develop only on exposure to the allergen and not after sensitization.<sup>66-68</sup>

Thus, murine models have contributed to our understanding of the mechanism of development and pathology of AAD. The use of knockout mice has certainly added value to our understanding of the roles of specific molecules and cytokines in these models. However, different strains of mice respond differently in these models displaying contrasting phenotypes in different backgrounds.<sup>69</sup> Comparative animal models of AAD using C57BL/6 and BALB/c backgrounds have also been reported.<sup>70</sup> Mouse models that replicate some of the features of asthma have been used extensively to try to uncover the underlying mechanisms of disease.<sup>71-74</sup>

T helper 2 (Th2) responses are associated with mouse models of asthma and human asthma by production of cytokines contributing to the infiltration of eosinophils (IL-5, eotaxin), mucus hypersecretion (IL-13) and IgE production (IL-4), with IL-13 being associated with both allergic and non-allergic asthma.<sup>75-81</sup> These mouse models have demonstrated that the IL-4R $\alpha$  and its ligands IL-4 and IL-13 are important in the induction of Th2-driven airway inflammation, mucus hypersecretion and airway hyperreactivity.<sup>74, 82-84</sup>

In effect this demonstrates that the mechanisms behind asthma pathogenesis are not fully understood. Individual cytokines have a wide range of effects which may be beneficial or deleterious depending upon the cells through which they signal. By determining the cell specific effects of cytokine signalling, we will be able to uncover the pathways that are important for the elicitation of airway disease and identify downstream effector molecules that may be important therapeutic targets.



**Figure 3. Immunological mechanism of asthma.** Progression of asthma can involve three phases. In acute phase (a) allergen-induced activation of mast cells releases pro-inflammatory cytokines and mediators, leading to acute bronchoconstriction and airway obstruction, which leads to the chronic phase (b) characterized by activation of Th2 cells and macrophages, recruitment and degranulation of eosinophils leading to changes in the airway causing airflow obstruction and increase in airway responsiveness. This can progress further to remodelling changes (c) leading to permanent irreversible alterations in the airway architecture. Figure from James E Gern et al, 2002.<sup>85</sup>

### 1.3 Immunological mechanisms of AAD

AAD, an inflammatory disease is caused by a Th2 type immune response. The immediate reactions of AAD are mediated by the release of inflammatory mediators by IgE. However, the mechanisms of late and chronic phases of AAD are poorly understood. A complex interaction of immune cells, cytokines and resident cells cause the development of disease symptoms of AAD where Th2 cytokines play a central role in this processes. IL-4 is critical for the development of Th2 cells from uncommitted Th0 cells and is also important for isotype switching of B cells from IgG to IgE. Mice deficient in IL-4 showed abrogated Th2 response.<sup>86</sup> IL-4 neutralization during the sensitisation phase caused a reduction in airway hyperresponsiveness, eosinophilia and allergen-specific IgE production.<sup>19</sup> However, IL-4 neutralisation during the effector phase did not have an effect on AAD.<sup>45</sup>

IL-13 produced by Th2 cells, mast cells, basophils and eosinophils promotes development of alternatively activated macrophages that have been hypothesized to play a role in allergies.<sup>875</sup> Studies have shown that main symptoms of allergic asthma were induced by IL-13 independently of IL-4 and IL-13 is mainly responsible for goblet cell hyperplasia and mucus production<sup>19, 75</sup> The induction of AHR by IL-13 through a direct effect on contraction of smooth muscle cells of the airways has also been reported.<sup>88</sup> Murine studies have also confirmed a common signalling pathway ie IL-4R $\alpha$  for both IL-4 and IL-13.<sup>89</sup>

Besides IL-4 and IL-13, there are other cytokines that contribute to the development of AAD. IL-5 plays a significant role in AAD through inducing eosinophils at inflammation sites, since it is critically involved in the growth, differentiation, maturation, recruitment and activation of eosinophils.<sup>90-93</sup> The role of IL-5 in the pathogenesis of asthma has been supported by various lines of experimental evidence. IL-5 deficient mice showed impaired numbers of eosinophils in the periphery and the lung after allergen challenge.<sup>45, 94-97</sup> In Clinical trials of asthmatic patients, a humanized blocking antibody specific for IL-5 (mepolizumab) although showed significant reduction in eosinophils had no effect on improving the disease.<sup>98-101</sup>

In mice, IL-9 blockade inhibited pulmonary eosinophilia, mucus hypersecretion, and AHR after allergen challenge of sensitized mice. Studies in asthmatic patients have also shown the

increased expression of IL-9 and its receptor in the airways enhancing mucus production, eosinophilia and AHR.<sup>101, 102</sup>

The functional role of IL-17 in asthma is unclear, since studies have shown a dual role for IL-17. IL-17 seems to be involved in allergic sensitization of animal models by inhibiting eosinophilic inflammation in sensitized animals and is known to induce proinflammatory cytokines contributing to acute inflammation.<sup>103</sup> Murine models have shown that IL-25 (IL-17E) produced by Th2 cells, mast cells, and epithelial cells induces the expression of IL-4, IL-5, and IL-13, resulting in inflammation mediated by eosinophils, increased IgE production, and AHR in mice.<sup>104</sup> Expression of IL-17 in the effector phase of allergic asthma reduces allergic symptoms by inhibiting dendritic cell functions and expression of IL-4 and IL-5.<sup>105</sup> Th17 cells are characterized by IL-17 (or IL-17A), IL-17F, IL-6, TNF- $\alpha$ , and IL-22 expression. IL-17A and IL-17F release neutrophil chemoattractants CXCL1 and CXCL8 from airway epithelial cells and airway smooth muscle cells and are responsible for neutrophil-mediated inflammation.<sup>106</sup> Thus IL-17 plays an essential role in the neutrophilic inflammation of severe asthma. Increased expression of mucin-encoding genes (*MUC5AC* and *MUC5B*) in human airway epithelial cells by IL-17 has also been reported.<sup>107</sup> Human studies have also shown the increased levels of IL-17A in the sputum of individuals with asthma and an increase in Th17 cells in the airways.<sup>108, 109</sup> Human studies have also confirmed that the secretion of Th2 cytokines is enhanced by IL-25.<sup>110, 111</sup> Blocking of IL-25 in mice inhibited the development of AHR in response to allergens.<sup>112</sup>

## **1.4 IL-4 Receptor alpha (IL-4R $\alpha$ ): common receptor for IL-4 and IL-13**

The fundamental Th2-associated cytokines, IL-4 and IL-13, play a key role in allergic sensitisation and in the development of symptoms for allergy as established by a range of experimental models and epidemiological studies.<sup>113</sup> The genes for both these cytokines are closely linked on both human and mouse chromosomes.<sup>114</sup>

### **1.4.1 IL-4 and IL-13 Receptors**

IL-4 and IL-13 have similar tertiary structures even though their amino acid homology amino is low.<sup>115</sup> IL-4 and IL-13 show overlapping functions, which is partly due to a shared common receptor transmembrane subunit, interleukin-4-receptor alpha (IL-4R $\alpha$ ), in their receptor complexes as shown in figure 4.<sup>116, 117</sup>

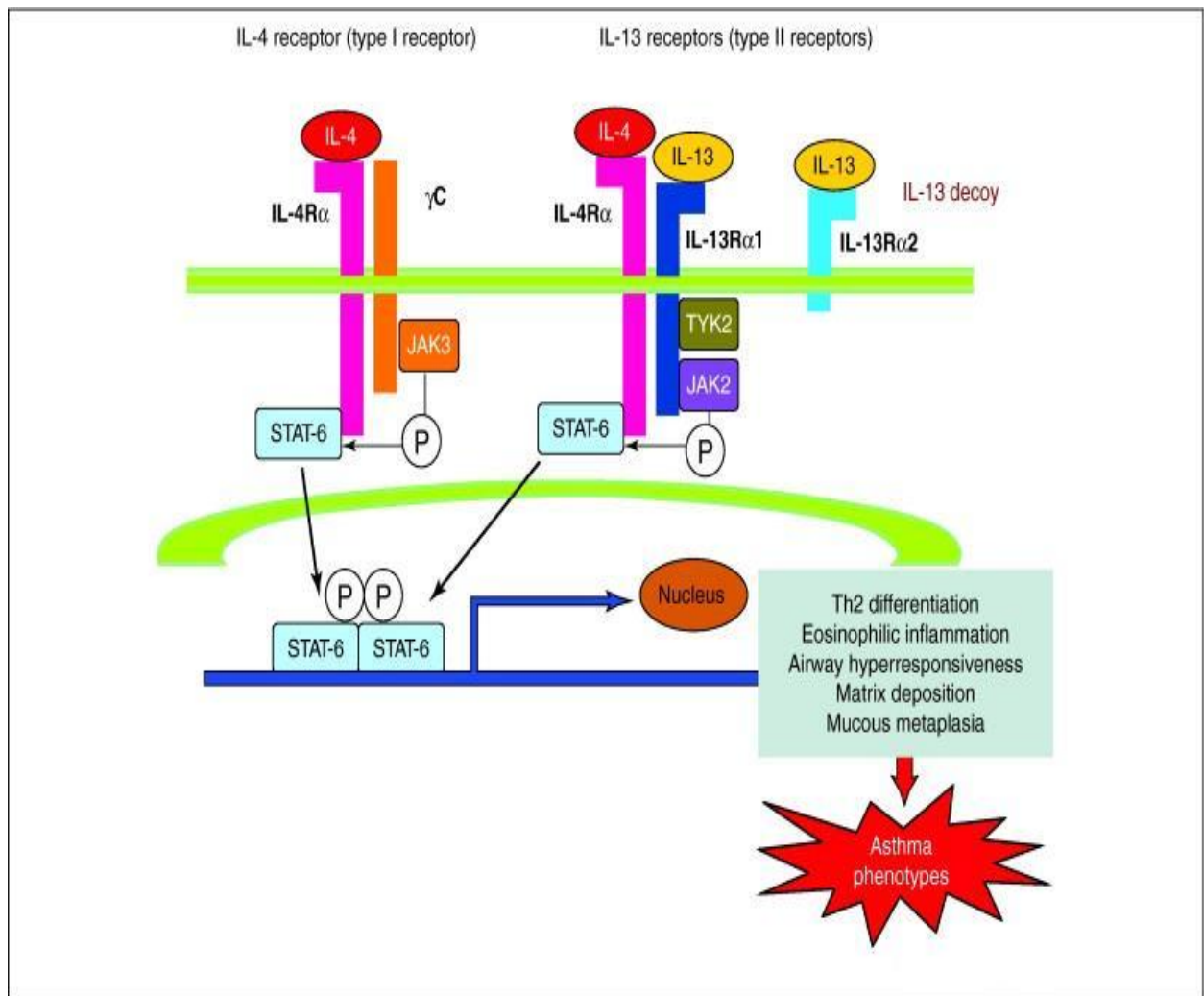
IL-4 receptor is a type I transmembrane protein that can bind both IL-4 and IL-13 and has various functions. The IL-4R $\alpha$  on T cells binds IL-4 promoting differentiation of Th2 cells. Allelic variations in this gene have been associated with many allergic conditions such as atopy, asthma, eczema and, sinusitis.<sup>116</sup> It is also important for the inhibition of Th1-associated pro-inflammatory cytokines such as IFN $\gamma$  and TNF.<sup>118</sup> Mice treated with anti-IL-4R $\alpha$  antibodies specifically blocked responses of IL-4 signalling.<sup>119-122</sup> The IL-4R $\alpha$  consists of a 140-kDa IL-4R $\alpha$  chain and is a component of both the type I and type II IL-4 receptors. It is expressed in relatively low numbers on numerous cell types.<sup>124, 125</sup> The type I IL-4 receptor is a combination of IL-4R $\alpha$  with the common  $\gamma$ -chain ( $\gamma$ c) of cytokine receptors, which is shared with other cytokine receptors, such as IL-2, IL-7, IL-9, IL-15 and IL-21.<sup>125,126</sup> The IL-4R $\alpha$  subunit paired with the 65-70kDa IL-13R $\alpha$ 1 chain forms a complex known as type II IL-4R $\alpha$  or IL-13 receptor, through which both IL-4 and IL-13 can signal. The IL-13R $\alpha$ 1 has a low binding affinity to IL-13 but when paired with IL-4R $\alpha$ , it has a high affinity for IL-13. IL-4 signalling can occur through both type I and type II receptors whereas IL-13 only binds to type 2 receptors.<sup>126,127</sup> IL-13R $\alpha$ 1 is expressed on many cell types including- B cells, basophils, endothelial cells, macrophages, mast cells and monocytes.<sup>126, 128,129</sup>

Although IL-13 signals via the IL-13R $\alpha$ 1, studies have shown that IL-13 has a higher binding affinity to the  $\alpha$ 2 chain of the IL-13 receptor (IL-13R $\alpha$ 2) and can signal through it.<sup>130</sup> IL-13R $\alpha$ 2 is a 55-60 kDa protein closely related to IL-13R $\alpha$ 1 except that the cytoplasmic domain has no signalling motifs or binding sequences for signalling molecules.<sup>131, 132</sup> In addition to the cell surface receptors, soluble forms of both IL-4R $\alpha$  and IL-13R $\alpha$ 2 exist. These are suggested to bind IL-4 and IL-13 with high affinity as non-signalling monomers. Therefore, the soluble receptors can act as competitive inhibitors of IL-4 and IL-13 regulating their activity.<sup>133, 134</sup>

IL-4R $\alpha$  brings together receptor associated Janus Kinases (JAKs) upon binding of IL-4 to IL-4R $\alpha$  or binding of IL-13 to IL-4R $\alpha$ /IL-13R $\alpha$ 1 complex as shown in figure 1.4. JAK-dependent tyrosine phosphorylation of the IL-4R $\alpha$  results in phosphorylation of the transcription factor known as signal transducer and activator of transcription 6 (STAT 6) enabling the transcription of many Th2 associated genes by translocation to the nucleus.<sup>135,136</sup> Other signalling intermediates like Insulin like receptor substrate (IRS) family can also be phosphorylated by IL-4R $\alpha$ . This common signalling pathway favours the sharing of many biological functions of IL-4 and IL-13 such as induction of B-cell isotype switching to IgE,

alternate macrophage activation, inhibition of inflammatory macrophage products, upregulation of MHC class II and promotion of Th2 type responses.<sup>113, 115, 137</sup> Different affinities to the shared type II receptor complex for both IL-4 and IL-13 have also been reported.<sup>138</sup>

IL-4 and IL-13 are predominantly produced by basophils, mast cells and activated CD4<sup>+</sup> Th2 cells. In spite of common functions, these two cytokines have distinct functions because of the expression of various receptor components on different cell types. T cells do not express type II IL-4 receptor (IL-13R $\alpha$ 1) and hence cannot respond to IL-13. This indicates the dependency of Th2 cell differentiation on IL-4 but not IL-13 signals. Human and mouse B cells respond differently to IL-13 for IgE class switch. Type I and type II IL-4 receptors are widely expressed in different cell types and tissues. A high affinity receptor for IL-13 is IL-13R $\alpha$ 2 which exists in membrane bound and soluble forms. Due to the lack in function of intracellular signalling, IL-13R $\alpha$ 2 has been reported as a decoy receptor which also inhibits IL-13 dependent symptoms in experimental asthma providing a negative feedback mechanism in allergic diseases.<sup>120, 139-141</sup>



**Figure 4. IL-4, IL-13 cytokines, their receptor complexes and general signalling pathways.** The IL-4 cytokine can bind to a type I (left) or Type II (middle) receptor, while IL-13 can bind to the Type II (middle) heterodimeric receptor or IL-13Rα2 (right). The IL-4Rα subunit is shared in both receptor types and associates with either JAK which activates STAT6 upon binding, or with Syk to induce intracellular signalling. Figure from Holgate 2012.<sup>142</sup>



### 1.4.2 IL-4R $\alpha$ in allergies and asthma

Allergen specific Th2 cells inducing symptoms of allergic airway disease have been reported.<sup>143, 144</sup> Studies have shown that CD4<sup>+</sup> T cells are necessary for the development of AHR and airway eosinophilia.<sup>19</sup> The key cells contributing to the onset and pathogenesis of asthma include T cells, B cells, epithelial cells and smooth muscle cells all of which express functional IL-4R $\alpha$ .

Experimental models in which IL-4R $\alpha$  was blocked, showed impairment in the development of AHR and airway eosinophilia.<sup>145, 146</sup> Mice deficient in IL-4R $\alpha$  lack allergen specific IgE production, eosinophilic airway inflammation and goblet cell hyperplasia and have abrogated Th2 type responses.<sup>75, 147</sup> IL-4R $\alpha$  deficient animal models have also shown antigen specific Th1 type cytokine and antibody responses and airway inflammation characterised by an increase in number of neutrophils. Other studies using gene deficient mice have shown that the development of AHR and specific IgE is IL-5 and IL-13 dependent.<sup>148</sup> Development of IgE and allergic responses after prolonged sensitization in IL-4R $\alpha$ <sup>-/-</sup> has also been demonstrated.<sup>149, 150</sup> Our laboratory has also shown that IL-4R $\alpha$  on smooth muscle cells is not required for the development of experimental allergic asthma.<sup>151</sup> However, IL-4R $\alpha$  signalling on smooth muscle cells can contribute to disease. IL-4 and IL-13 responsiveness may also contribute to the development of a Th2 type immune response.<sup>152, 153</sup> IL-4 and IL-13 signalling through IL-4R $\alpha$  induces the development of alternatively activated macrophages.<sup>87</sup>

IL-4, IL-13 and their receptors are potential drug targets for allergic asthma as the pathology of the disease involves these IL-4R $\alpha$  expressing cells. Although the use of anti IL-4 monoclonal antibodies was not effective at preventing asthma, the use of soluble IL-4R $\alpha$  antagonists did show some convincing results.<sup>154, 155</sup> Clinical studies have also established that Single Nucleotide Polymorphisms (SNPs) in IL4R $\alpha$  are associated with severe asthma exacerbations, lower lung function, and increased mast cell-related tissue inflammation.<sup>156</sup> Furthermore, another clinical study has investigated the conservative region of IL-4R $\alpha$  in human susceptibility to allergic asthma, highlighting the importance of IL-4R $\alpha$  in allergic asthma.<sup>157</sup>

Thus IL-4R $\alpha$  signalling seem to be essential for the development of Th2 cells in allergic airway disease but the contribution of IL-4R $\alpha$  dependent Th2 cells (or: IL-4 and IL-13 responsive Th2 cells) to the disease development yet to be characterised.

### 1.4.3 Cell specific knockout mice and IL-4R $\alpha$ knockout mouse models

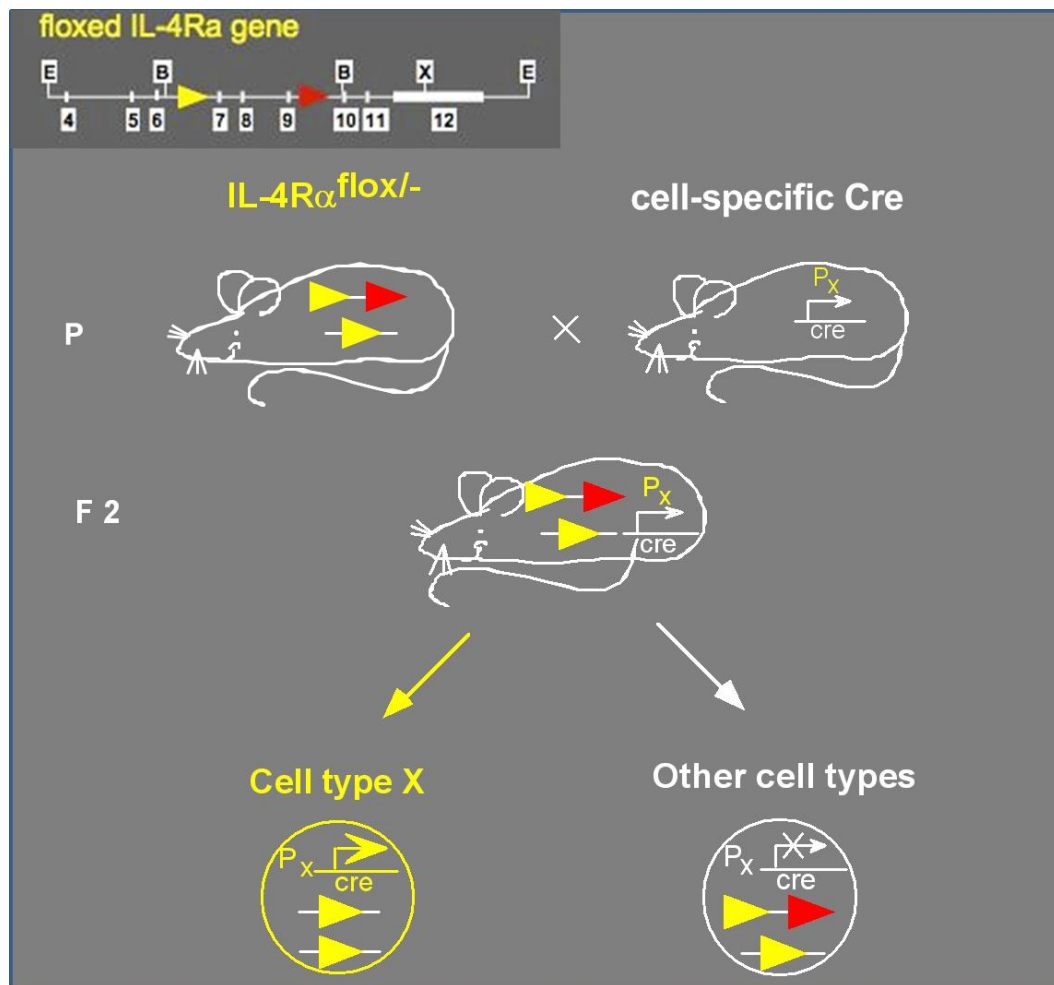
The Cre recombinase-loxP (Cre-loxP) system has led to the development of several transgenic strains of mice that enable the investigation of cell specific gene function *in vivo*.<sup>158-160</sup> This system utilises enzymes and DNA sequences derived from bacteriophage P1, which uses them to excise itself from the host cell. Integration of bacteriophage P1 DNA into the genome of the host cell results in flanking of the targeted gene promoter by loxP sites, which are recognised by the enzyme Cre recombinase. Introduction of loxP sites on either side of a gene or exon in the mouse favour the excision of these specific genes or exons, resulting in cell specific deletion. Mice containing loxP sites flanking a specific gene or exons are mated with transgenic mice that express Cre recombinase under the control of promoter of the type of cell or tissue of interest. Thus the active Cre-recombinase in the appropriate tissue will excise the DNA between the inserted loxP sites effectively inactivating the targeted gene. Cells lacking the expression of the Cre- recombinase promoter will not excise the gene thus resulting in a tissue specific knockout mouse.<sup>161</sup>

Using the Cre-loxP system as illustrated in figure 5, our laboratory and others have generated mice with cell-specific IL-4R $\alpha$  deletion (exon 7-9) in several different cell types, for e.g. specific impairment of IL-4R $\alpha$  expression on CD4<sup>+</sup> T-cells<sup>162-164</sup>, dendritic cells and alveolar macrophages, lung (Clara) cells<sup>82</sup>, macrophages and neutrophils<sup>165,166</sup>, B cells and smooth muscle cells<sup>151,167</sup>. These cell-specific IL-4R $\alpha$ -deficient mice have been used to study asthma<sup>166</sup>, anaphylaxis<sup>163</sup>, intracellular parasite and helminth infections<sup>162,167</sup> unravelling the importance of Th2 and hence IL-4R $\alpha$ -dependent mechanisms in these cell lineages. The first experimental study to demonstrate that IL-4R $\alpha$  signalling in a single cell type was sufficient for the development of a major symptom of allergic airway disease was performed by Kuperman *et al* that allergen induced mucus production is dependent on IL-4R $\alpha$  signalling in Clara cells.<sup>151,168</sup>

Generation of transgenic mice with cell type specific disruptions in the IL-4R $\alpha$  gene are an excellent tool for defining a role for specific contributions of IL-4R $\alpha$  signalling in these cells

in allergic airway disease. Although the general importance of the IL-4R $\alpha$  in disease development is well established, its cell type specific functions often remain elusive. To date the cell specific role of IL-4R $\alpha$  in allergy has only been described *in vivo* for epithelial cells and smooth muscle cells. Disrupted IL-4R $\alpha$  signalling on epithelial cells resulted in protection from airway mucus production but not airway inflammation or hyperreactivity<sup>82</sup> whereas the impairment of IL-4R $\alpha$  on smooth muscle cells in early stages of AAD did not result in disease protection.<sup>151</sup>

However it is quite crucial to determine the cell specific effects of cytokine signalling so that the pathways important for the elicitation of AAD could be defined. This would eventually lead to the identification of downstream effector molecules that may serve as important therapeutic targets.



**Figure 5. Breeding strategy for generation of cell specific IL-4R $\alpha$  knockout mice.** Mice heterozygous for a floxed and deleted IL-4R $\alpha$  allele were crossed with mice harboring the Cre-recombinase gene under the control of a cell specific promoter. The Cre, expressed only in the desired cell type, recognizes the floxed sequence and excises the portion of the gene in between resulting in a cell specific deletion of the IL-4R $\alpha$  gene. Using a heterozygous mouse increases the efficiency of deletion by reducing the substrate for the Cre-recombinase by half. The insert in the figure shows the IL-4R $\alpha$  gene and restriction enzyme sites are labelled E, B and X. Figure from Radwanska et al 2007.<sup>164</sup>

#### 1.4.3.1 CD4<sup>+</sup> T cell specific IL-4R $\alpha$ -deficient mice (Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup>)

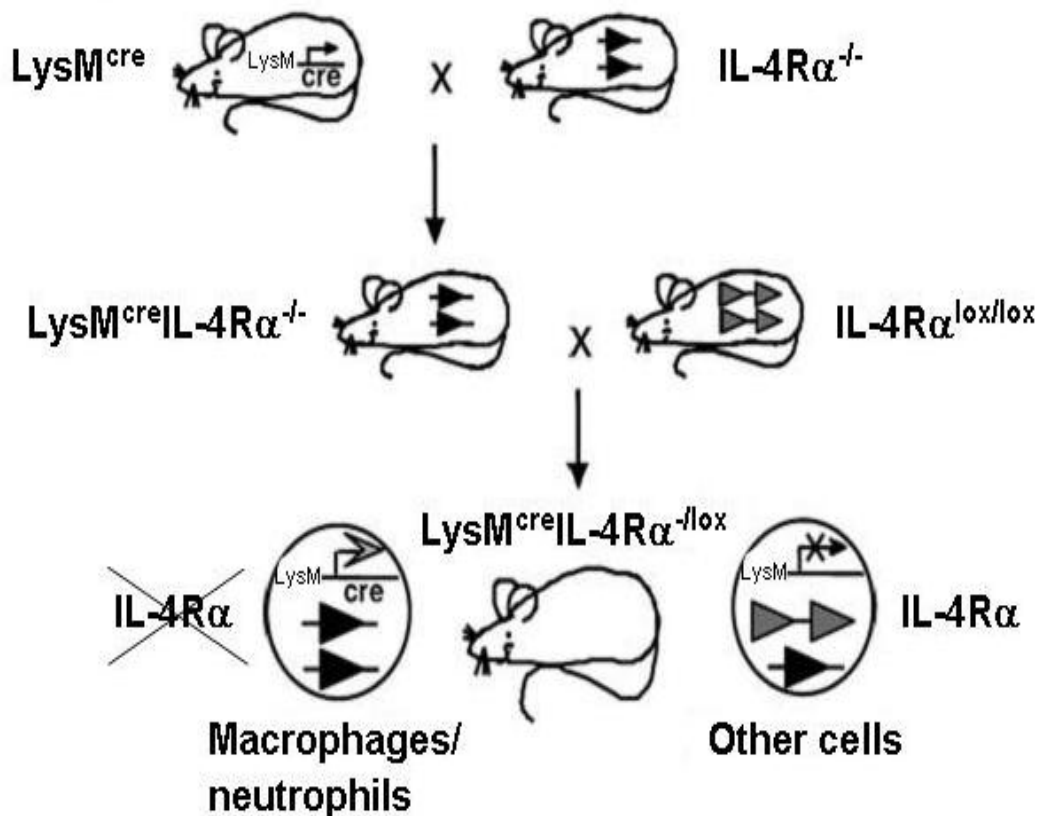
Using the principles described above in Figure 5, mice with a specific deletion of IL-4R $\alpha$  on CD4<sup>+</sup> T cells have been generated.<sup>164</sup> An IL-4R $\alpha$ <sup>-/-</sup> mouse was crossed with a transgenic mouse expressing Cre recombinase under control of the T-cell specific promoter, lymphocyte specific tyrosine kinase (Lck<sup>cre</sup>) to produce a Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-/-</sup> mouse. Subsequently, the Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-/-</sup> mouse was crossed with a mouse bearing two loxed IL-4R $\alpha$  alleles (lox/lox) in order to produce Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> offspring. IL-4R $\alpha$  hemizyosity (<sup>-lox</sup>) increases the probability of Cre-mediated deletion of the loxed allele. To generate Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice with a BALB/c background, Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice were back crossed to BALB/c wild types for 9 generations. To confirm the specificity and efficiency of IL-4R $\alpha$ <sup>-/-</sup> deletion on CD4<sup>+</sup> T cells PCR genotyping, FACS analysis and real time PCR were performed.

The Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mouse strain, characterized by complete deletion of IL-4R $\alpha$  on CD4<sup>+</sup> T showed incomplete deletion efficiency on CD8<sup>+</sup> and NK T cells and normal IL-4R $\alpha$  expression on CD4<sup>+</sup> T cells. These mice have impaired IL-4-induced CD4<sup>+</sup> proliferation and Th2 differentiation, but normal IL-4 and/or IL-13 responsiveness by other T cells and non-T cells.<sup>82</sup> Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice were found to be protected against anaphylaxis in a manner that was dependent upon their increased production of IFN-gamma.<sup>163</sup> They were also protected against *Leishmania major* infection<sup>164</sup>, had increased survival after infection with *Schistosoma mansoni*<sup>169</sup> and were protected in oxazolone-induced colitis.<sup>170</sup> Furthermore, Lck<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice showed reduced lung pathology after *Nippostrongylus brasiliensis* infection, with decreased mucus secretion, lymphocytes and eosinophils in the lungs.<sup>162</sup>

#### 1.4.3.2 Macrophage/neutrophil specific IL-4R $\alpha$ -deficient mice (LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup>)

Mice with a specific deletion of IL-4R $\alpha$  on macrophage/neutrophil have been generated.<sup>164</sup> An IL-4R $\alpha$ <sup>-/-</sup> mouse was crossed with a transgenic mouse expressing Cre recombinase under the control of a Lysozyme M promoter to produce a LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-/-</sup> mouse. Subsequently, the LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-/-</sup> mouse was crossed with a mouse bearing two loxed IL-4R $\alpha$  alleles (lox/lox) in order to produce LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> offspring. IL-4R $\alpha$  hemizyosity (<sup>-lox</sup>) increases the probability of Cre-mediated deletion of the loxed allele. To generate LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice with a BALB/c background, LysM<sup>cre</sup> IL-4R $\alpha$ <sup>-lox</sup> mice were back crossed to BALB/c wild types for 9 generations.

Genes under the macrophage- and neutrophil-specific lysozyme M (LysM) promoter<sup>171</sup> have also been used to investigate Arginase1 ( $Arg1^{-/lox}$ ; LysMCre)<sup>172</sup> or IL-4R $\alpha$  (LysM<sup>Cre</sup>IL-4R $\alpha^{-/lox}$ ) function in macrophage/neutrophil lineages in helminth infections<sup>162,167</sup>, anaphylaxis<sup>163</sup>, arthritis<sup>173</sup> and colon carcinoma<sup>174</sup>. LysM<sup>Cre</sup>IL-4R $\alpha^{-/lox}$  mice were generated as shown in Figure 6 and characterized.



**Figure 6. Generation of  $LysM^{Cre}IL-4R\alpha^{-/lox}$  mice.**  $IL-4R\alpha^{-/-}$  mice were mated with transgenic mice possessing a Lysozyme M promoter driving Cre recombinase, resulting in  $LysM^{Cre}IL-4R\alpha^{-/-}$  offspring. These offspring were mated with mice containing IL-4R $\alpha$  flanked by loxP sites ( $IL-4R\alpha^{lox/lox}$ ) to produce mice with LysM driven cre-mediated deletion of IL-4R $\alpha$  ( $LysM^{Cre}IL-4R\alpha^{-/lox}$ ). Figure from Herbert et al 2004.<sup>165</sup>

## 1.5 Thesis Objectives

The aim of this study is to investigate the cell specific contributions of IL-4R $\alpha$  signalling in the development of Ovalbumin-induced chronic allergic airway disease (AAD) in mice.

Specifically, we aim to investigate the following:

Firstly, the role of IL-4/IL-13 responsive CD4<sup>+</sup> T cells in AAD, using Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice and

Secondly, the role of IL-4/IL-13 induced alternatively activated macrophages (AAM) in AAD, using LysMcreIL-4R $\alpha$ <sup>-lox</sup> mice.

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## **CHAPTER 2**

**IL-4R $\alpha$  expression on CD4<sup>+</sup> T cells is required for  
chronic allergic airway disease (AAD) in mice**

## 2 IL-4R $\alpha$ expression on CD4<sup>+</sup> T cells is required for chronic allergic airway disease (AAD) in mice

### 2.1 Summary

#### Background

To date the cell specific role of IL-4R $\alpha$  in AAD has only been described *in vivo* for epithelial cells and smooth muscle cells. Disrupted epithelial IL-4R $\alpha$  signalling resulted in decreased airway mucus production but did not affect airway inflammation or hyperreactivity. This study was conducted to investigate how IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells influences the development of AAD. By using transgenic Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice deficient for IL-4R $\alpha$  on CD4<sup>+</sup> T cells, the *in vivo* effects of impaired IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells on the outcome of AAD could be investigated.

#### Objective

The aim of this study is to investigate the chronic effects of AAD in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice.

#### Methods

AAD was introduced in mice by subcutaneous sensitisation with 20  $\mu$ g ovalbumin (OVA) adsorbed to 0.65% alum on days 0, 7, 14, and 21. On days 27 and 29 and then twice weekly for 4 weeks, mice were challenged intranasally with 20  $\mu$ g OVA in 50  $\mu$ L PBS under anaesthesia. Mice were investigated for the presence of airway hyperresponsiveness (AHR), markers of allergic inflammation, allergen specific Th2 type antibody and cytokine production.

#### Results

Our studies using the chronic murine model of AAD established that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice were significantly protected. Airway hyperreactivity, Th2 responses, mucus hypersecretion, eosinophil infiltration, and collagen deposition were decreased in comparison to IL-4R $\alpha$ <sup>-lox</sup> littermate controls.

## Conclusion

These findings demonstrate that IL-4R $\alpha$  signalling through CD4<sup>+</sup> T cells is critical for the outcome of the chronic OVA-induced AAD in mice.

## 2.2 Introduction

Allergic asthma is characterised by inflammatory responses to inhaled allergens orchestrated by CD4<sup>+</sup> T cells which produce a characteristic Th2 cytokine profile causing eosinophilic lung inflammation.<sup>1</sup> Thus Th2 responses drive chronic allergic lung inflammation in asthmatic patients after allergen challenge by elevated numbers of CD4<sup>+</sup> T cells and Th2 cytokines in bronchoalveolar lavage (BAL) specimens.<sup>2</sup> Airway remodelling, a characteristic of chronic asthma is a consequence of unresolved airway inflammation that results in irreversible permanent structural changes in the airways. The structural changes include: increased airway wall thickness involving smooth muscle and collagen tissue, subepithelial fibrosis and hyperplasia of myofibroblasts and goblet cells<sup>3, 4, 5</sup> leading to increased mucus production and increased vascularity in the airways contributing to sustained AHR.<sup>6,7,8</sup> These structural changes lead to reduced lung function in asthmatic patients and airway biopsies from these patients suggest that the disease severity<sup>9</sup> and AHR is related to both the degree of smooth muscle thickness and the extent of subepithelial fibrosis and magnitude of AHR.<sup>10,11</sup> In patients with atopic asthma, allergen inhalation challenge lead to activation of CD4<sup>+</sup> T cells and increases Th2-type cytokine mRNA expression, which is thought to contribute to late asthmatic responses by mechanisms that include eosinophil accumulation in the airways.<sup>12</sup>

Murine models of acute AAD have shown that CD4<sup>+</sup> T cells are required for eosinophilic lung inflammation and airway hyperresponsiveness, and adoptively transferred CD4<sup>+</sup> T cells can induce these features.<sup>13, 14</sup> In mice, CD4<sup>+</sup> T cells contribute to the pathology similar to human asthma by an IL-4 dependent mechanism.<sup>15</sup> Studies using murine models have also shown that Th2 cytokines which are produced as a result of CD4<sup>+</sup> T cells can induce AHR at an early stage without a requirement for inflammatory cell recruitment.<sup>16</sup> It has also been established that Th2-induced airway mucus production is dependent on IL-4R $\alpha$ , but not on eosinophils.<sup>17</sup> However, therapies targeting either CD4<sup>+</sup> T cells or Th2 cytokines such as IL-4

and IL-5 have not been successful.<sup>18-20</sup> The mechanisms through which CD4<sup>+</sup> T cells contribute to chronic asthma pathogenesis remains poorly understood.

In acute murine models of AAD, CD4<sup>+</sup> T cell depletion reduced inflammation and airway remodelling.<sup>21, 22</sup> Furthermore, adoptively transferred CD4<sup>+</sup> T cells from ovalbumin (OVA)-sensitized rats induced increases in airway smooth muscle mass after repetitive challenge with OVA via the airways.<sup>23</sup> Thus, CD4<sup>+</sup> T cells appear to be required for both initiation of airway inflammation and induction of remodeling. CD4 depletion three weeks after the termination of chronic antigen challenge was found to have no effect on features of airway remodeling.<sup>24</sup> Even though still underwent the process of lung remodelling, CD4<sup>+</sup> T cell depletion in mice displayed significant reduction in signs of lung inflammation.<sup>25</sup>

The Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mouse strain is characterized by complete deletion of IL-4Rα on CD4<sup>+</sup> T cells, incomplete deletion efficiency in CD8<sup>+</sup> and NK T cells and normal IL-4Rα expression on γδ<sup>+</sup> T cells.<sup>26</sup> These mice have impaired IL-4-induced CD4<sup>+</sup> proliferation and Th2 differentiation, but normal IL-4 and/or IL-13 responsiveness by other T cells and non-T cells.<sup>26, 27</sup> Previous studies in our laboratory have shown the various roles for IL-4 responsive T cells in Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice. Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice were found to be protected against anaphylaxis in a manner that was dependent upon their increased production of IFN-γ.<sup>28</sup> They were also protected against *Leishmania major* infection,<sup>26</sup> had increased survival after infection with *Schistosoma mansoni*<sup>29</sup> and were protected in oxazolone-induced colitis.<sup>30</sup> Furthermore, Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice showed reduced lung pathology after *Nippostrongylus brasiliensis* infection, with decreased mucus secretion, lymphocytes and eosinophils in the lungs.<sup>31</sup>

The phenotype of Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice in a short-term model of acute allergic airway disease (AAD)<sup>32, 33</sup> was previously examined by another researcher in our laboratory. Despite all the evidence of the significance of Th2 in asthma, interestingly Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice maintained susceptibility to AAD in the acute model, with comparable airway hyperreactivity, airway resistance and elastance to IL-4Rα<sup>-/lox</sup> littermate control mice.<sup>34</sup> This susceptible phenotype was attributed to IL-4 independent IL-13 production by CD4<sup>+</sup> T cells, as Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> x IL-13<sup>-/-</sup> mice had abrogated allergic airway disease while Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> x IL-4<sup>-/-</sup> did not. Importantly this indicates a role for IL-4 independent Th2 cells in acute allergic airway disease. Hence we were interested to study the role of IL-4Rα

on CD4<sup>+</sup> T cells and airway remodelling in a chronic model. The role of IL-4 independent Th2 in allergic airway disease is of interest, and may explain why IL-4 neutralization does not work well as a therapy for asthma.

Therefore, we aimed to determine whether IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells contribute to ovalbumin induced AAD in a chronic model, and to examine the effect of CD4<sup>+</sup> T cell specific deletion of IL-4R $\alpha$  on airway remodelling. In contrast to the susceptibility of Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice in acute AAD, our studies using the long-term chronic model of AAD we have found that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice were significantly protected, showing decreased airway hyperreactivity, airway resistance, and airway elastance, as well as decreased numbers and percentages of eosinophils in the bronchiolar lavage fluid and lungs and decreased lung inflammation, mucus hypersecretion and airway remodelling compared to IL-4R $\alpha$ <sup>-lox</sup> littermate controls. Our results demonstrate significant reduction in AAD in the absence of IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells in the chronic model, suggesting that IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells may be critical in sustaining AAD in mice showing that IL-4R $\alpha$  signalling through CD4<sup>+</sup> T cells plays an important role in chronic AAD in mice.



## 2.3 Materials and Methods

### Mice

Eight- to ten week-old  $Lck^{cre}IL-4R\alpha^{-/lox}$ <sup>27</sup>,  $IL-4R\alpha^{-/lox}$ , and  $IL-4R\alpha^{-/-}$  female mice<sup>35</sup> on a BALB/c background were housed in independently ventilated cages under specific pathogen-free conditions in the University of Cape Town Animal Facility. Animal procedures were approved by the University of Cape Town Animal Ethics Committee. (Permit Number: 008/016)

### Generation of IL-4R $\alpha$ knockout mice

The generation and characterization of CD4<sup>+</sup> T cell-specific IL-4R $\alpha$ -deficient ( $Lck^{cre}IL-4R\alpha^{-/lox}$ ) mice were previously described.<sup>27</sup>  $Lck^{cre}$  mice on a BALB/c background were crossed with  $IL-4R\alpha^{-/-}$  mice<sup>36</sup> and  $IL-4R\alpha^{lox/lox}$  mice<sup>37</sup> to generate  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice.  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice were generated by mating floxed  $IL-4R\alpha^{lox/lox}$  mice with transgenic  $Lck^{cre}$  mice. These mice were then back-crossed to the BALB/c background for 9 generations prior to crossing with global IL-4R $\alpha$  knockout ( $IL-4R\alpha^{-/-}$ ) mice to finally obtain  $Lck^{cre}IL-4R\alpha^{-/lox}$  BALB/c mice. BALB/c ( $IL-4R\alpha^{+/+}$ ), transgene negative littermates ( $IL-4R\alpha^{-/lox}$ ) and  $IL-4R\alpha^{-/-}$  mice were used as controls in all experiments.

### Genotyping of IL-4R $\alpha$ knockout mice

Mice were genotyped by PCR to determine what IL-4R $\alpha$  allele was present and the PCR conditions were as follows: 94°C/30 s; 57°C/30 s, 72°C/1 min for 40 cycles on an MJ thermocycler (Biozym, Hessisch, Oldendorf, Germany).

Primers used for PCR screening of the mice:

#### oLCK Cre Transgene allele

Cre LCK R: 5'- CAG GTA TGC TCA GAA AAC GCC TGG -3'  
Prox LCKpF: 5'- GAG GGT GGA ATG AAA CTC TCG GT -3'

#### IL-4R $\alpha$ Wildtype allele

Exon 7 forward1: 5'-TGA CCT ACA AGG AAC CCA GGC-3'  
Exon 8 reverse: 5'-CTC GGC GCA CTG ACC CAT CT-3'

#### IL-4R $\alpha$ Deleted allele

IL-4R $\alpha$  KO P1: 5' - GGC TGC TGA CCT GGA ATA ACC - 3'  
IL-4R $\alpha$  KO P2: 5' - CCT TTG AGA ACT GCG GGC T - 3'

### **Conditional IL-4R $\alpha$ LoxP allele**

IL-4R $\alpha$  KO P1: 5' - GGC TGC TGA CCT GGA ATA ACC - 3'

IL-4R Intron 6-Rev: 5' - GTT TCC TCC TAC CGC TGA TT - 3'

### **Sensitisation and challenge of mice**

Mice were sensitized subcutaneously with 200  $\mu$ l (20  $\mu$ g ovalbumin (OVA) adsorbed to 0.65% alum) on days 0, 7, 14, and 21. On days 27 and 29 and then twice weekly for 4 weeks, mice were administered intranasal challenges with 50  $\mu$ l (20  $\mu$ g OVA in 50 mL PBS) under anaesthesia.<sup>38</sup>

### **Measurement of Airway Hyperreactivity**

Airway resistance and elastance of the whole respiratory system (airways, lung chest wall) after challenge with increasing doses of acetyl-b-methylcholine (methacholine, Sigma-Aldrich, Aston Manor, South Africa) were determined by forced oscillation measurements as described<sup>36</sup> with a Flexivent system (SCIREQ, Montreal, Canada) by using the singlecompartment ("snapshot") perturbation. Differences in the dose-response curves were analyzed by repeated-measures ANOVA. Only mice with acceptable measurements for all doses (coefficient of determination >0.95) were included in the analysis.

### **Flow cytometry**

Bronchoalveolar lavage (BAL) cells were obtained by flushing the lungs of sacrificed mice once with 1 mL PBS/10% FCS.

Single-cell suspensions were prepared from lungs after digestion for 1 hour at 37°C in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, Calif) containing 13 mg/mL DNase I (Roche, Randburg, South Africa) and 50 U/mL collagenase IV (Sigma-Aldrich, Aston Manor, South Africa).

For intracellular cytokine staining, lung cells were incubated at 37°C for 4 hours with phorbol myristate acetate (Sigma-Aldrich) (50 ng/mL), ionomycin (Sigma-Aldrich) (250 ng/mL), and monensin (Sigma-Aldrich) (200 mM in IMDM/10% FCS). Cells were stained with CD3-fluorescein isothiocyanate (clone 145-2C11) and CD4-PerCP (clone L3T4) (BD Bioscience), and intracellular cytokines were stained with anti-IL-4 (clone 11B11), anti-IL-5 (clone TRFK5), anti-IFN-g (clone XMG 1.2), anti-IL-17 (clone TC11-18H10) (BD Bioscience),

anti-IL-13 (clone eBio 13A) (eBioscience, San Diego, Calif), and rat anti-mouse IgG1 isotype control (clone A85-1) (BD Bioscience) (all PE-labeled). Cells were acquired on a FACSCalibur machine (BD Immunocytometry Systems, San Jose, Calif), and data were analyzed by using Flowjo software (Treestar, Ashland, Ore).

For flow cytometry, the following antibodies were used: SiglecF-PE (clone E50-2440), CD11c-APC (clone HL3), CD4-PerCP (clone L3T4), with streptavidin (all BD Bioscience, Erembodegem, Belgium), CD3 FITC fluorescein isothiocyanate (clone 145-2C11) and GR-1-fluorescein isothiocyanate (clone RB68C5).

### **Sorting of alveolar macrophages**

Alveolar macrophages were sorted as CD11c<sup>+</sup>SiglecF<sup>+</sup> cells from the pooled lungs of OVA-sensitized and -challenged mice. Cells were sorted on a FACSVantage flow cytometer (BD Immunocytometry Systems, San Jose, Calif). Purity was determined by flow cytometry and checked by cyto-spin and staining with the Rapidiff Stain set (Clinical Diagnostics CC, Southdale, South Africa) and was at least 99%.

### **RNA extraction, cDNA synthesis and Quantitative RT-PCR**

Total RNA was extracted from sorted alveolar macrophages of >98% purity by using Tri reagent (Applied Biosystems, Carlsbad, Calif) according to the manufacturer's protocol and purified with RNeasy Microprep kit (Qiagen Hilden, Germany).

Sorted alveolar macrophages were lysed in 500 µl TriReagent® (Molecular Research, USA) and stored at -80 °C. To extract the RNA, thawed samples were incubated at room temperature for 5 min to ensure complete dissociation of nucleoproteins. To each sample, 0.1 ml of chloroform was added and samples were vortexed for 15 seconds and incubated at room temperature for a further 15 min prior to centrifugation at 12000 rpm for 15 min at 4°C. The RNA contained in the upper aqueous layer was purified using the miRNeasy Mini Kit (Cat. No. 217004, Qiagen, USA), RNeasy MinElute Cleanup Kit (Cat. No. 74204, Qiagen, USA) and the RNase-Free DNase Set (Cat. No. 79254, Qiagen, USA). RNA quantity was determined using a Nanodrop ND1000 (Thermo Scientific, USA).

cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche). Real-time quantitative PCR was performed on cDNA samples to determine gene expression levels of marker genes by using Lightcycler FastStart DNA MasterPLUS SYBR Green I reaction mix (Roche) on a lightcycler 480 II (Roche). Each reaction contained SYBR® Green Master Mix

(Cat. No. 04913850001, Roche, Germany), primers and cDNA template. Reactions were prepared in 96- well plates (Roche, Germany) and PCRs were performed in a LightCycler® 480 (Roche, Germany).

Primers for inducible nitric oxide synthase (iNOS), arginase, Ym-1, and FIZZ-1 have been previously described and are listed in Table 2.1<sup>37, 39, 40</sup> Gene expression levels were normalized according to the expression of the housekeeping genes ribosomal rs12 or HPRT.

**Table 2.1: RT-PCR primers and PCR conditions**

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing Temperature (°C)	Extension time (s)	Fluorescence acquisition temperature (°C)
<b>Ym-1</b>	GGGCATACCT TTATCCTGAG	CCACTGAAGT CATCCATGTC	60	12	80
<b>Fizz-1</b>	TCCCAGTGAA TACTGATGAG A	CCACTCTGGA TCTCCCAAGA	60	9	80
<b>Arg1</b>	CAGAAGAATG GAAGAGTCAG	CAGATATGCA GGGAGTCACC	60	10	80
<b>iNOS</b>	AGCTCCTCCC AGGACCACAC	ACGCTGAGTA CCTCATTGGC	60	19	85
<b>rs12</b>	GGAAGGCATA GCTGCTGGAG GT	CGATGACATC CTTGGCCTGA	60	10	80

### Histology and immunohistochemistry

Lungs were fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections were stained with haemotoxylin and eosin (H&E), periodic acid-Schiff (PAS) for mucus, Sirius red for collagen, Martius scarlet blue (MSB) for fibrin and Gordon and Sweets for reticulin (reticulin silver staining kit). Image analysis was performed on NIS Elements (Nikon Instruments, Tokyo, Japan). Lung sections from individual mice were assessed, and data from 3 experiments were pooled.

### **Antibody ELISAs**

Blood samples taken by tail vein bleeding or directly from mice after sacrificing were collected in plasma separator tubes (Microtainer™ SST, BD, USA). Samples were centrifuged for 20min at 6000rcf and stored at -80°C. OVA specific antibodies were measured in blood serum by endpoint titration ELISA. For OVA specific antibodies, 96 well ELISA plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl OVA in PBS (5µg/ml for IgG1, IgG2a, IgG2b or 1mg/ml for IgE). For total IgE ELISA, plates were coated with anti-mouse IgE antibody (clone 84.1C, 1/1000 dilution). Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Serial dilutions of serum samples in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated overnight at 4 °C. Purified recombinant mouse IgE (BD, USA) was used as a standard for total IgE ELISA, at a starting concentration of 1mg/ml. 50µl alkaline phosphatase conjugated goat anti-mouse isotype specific antibodies (Southern Biotechnology, USA) at a 1/1000 dilution in PBS/ 0.1% BSA were added to the wells and the plates were incubated for 3h at 37°C. Subsequently, plates were incubated with 50µl p-nitrophenylphosphate (Fluka, Switzerland) and the colour reaction was stopped with 50µl 1M NaOH. Absorption was measured at 405nm with 492nm as reference wave length using a VersaMax plate reader (Molecular devices, USA). Plates were washed 4x with 200µl per well after each incubation step.

### **Statistical analysis**

P values were calculated in GraphPad Prism 4 (GraphPad Software, Inc, San Diego, Calif) by using ANOVA with Bonferroni's posttest or repeated measures ANOVA, and results are presented as mean  $\pm$  SD of the mean. Values  $p < 0.05$  was considered significant.

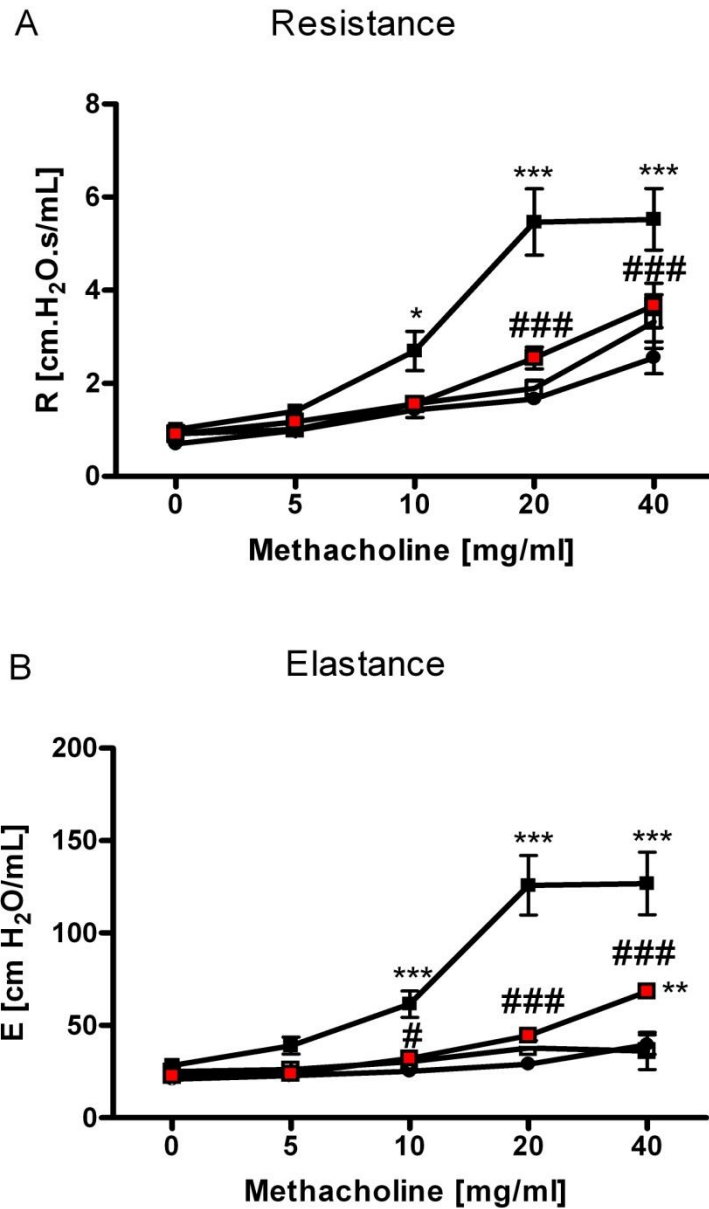
## 2.4 Results

### 2.4.1 $Lck^{cre}IL-4R\alpha^{-/lox}$ mice are protected against the development of AHR

The airway responses of  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice and littermate controls were examined in OVA-induced allergic airway disease. Airway resistance (R) and elastance (E) were measured in response to increasing doses of methacholine after chronic exposure to OVA. Airway dynamic resistance (R) quantitatively assesses the level of constriction in the lungs. Increased R values signal constriction of the lungs and this parameter reflects not only central airway resistance, but is also influenced by the lung periphery (the "tissues"). Dynamic elastance (E) captures the elastic rigidity of the lungs and reflects the mechanical properties of the lungs for normal respiratory rates. Increased E values signal an increased stiffness of the lungs.

The OVA sensitised and challenged mice developed AHR, with significant differences between  $IL-4R\alpha^{-/lox}$  PBS mice littermate controls and  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice (Fig.1 A, B). A trend toward a significant decrease in both airway resistance and elastance was observed in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice compared to littermate control mice. Global  $IL-4R\alpha^{-/-}$  mice did not show a significant increase in AHR compared with saline controls, demonstrating protection. Since airway resistance assesses constriction of the airways, an increase in E values may indicate similar airway remodeling in littermate controls. These results provide strong evidence that the absence of IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells protect against AHR.

## Airway Hyper Responsiveness (AHR)



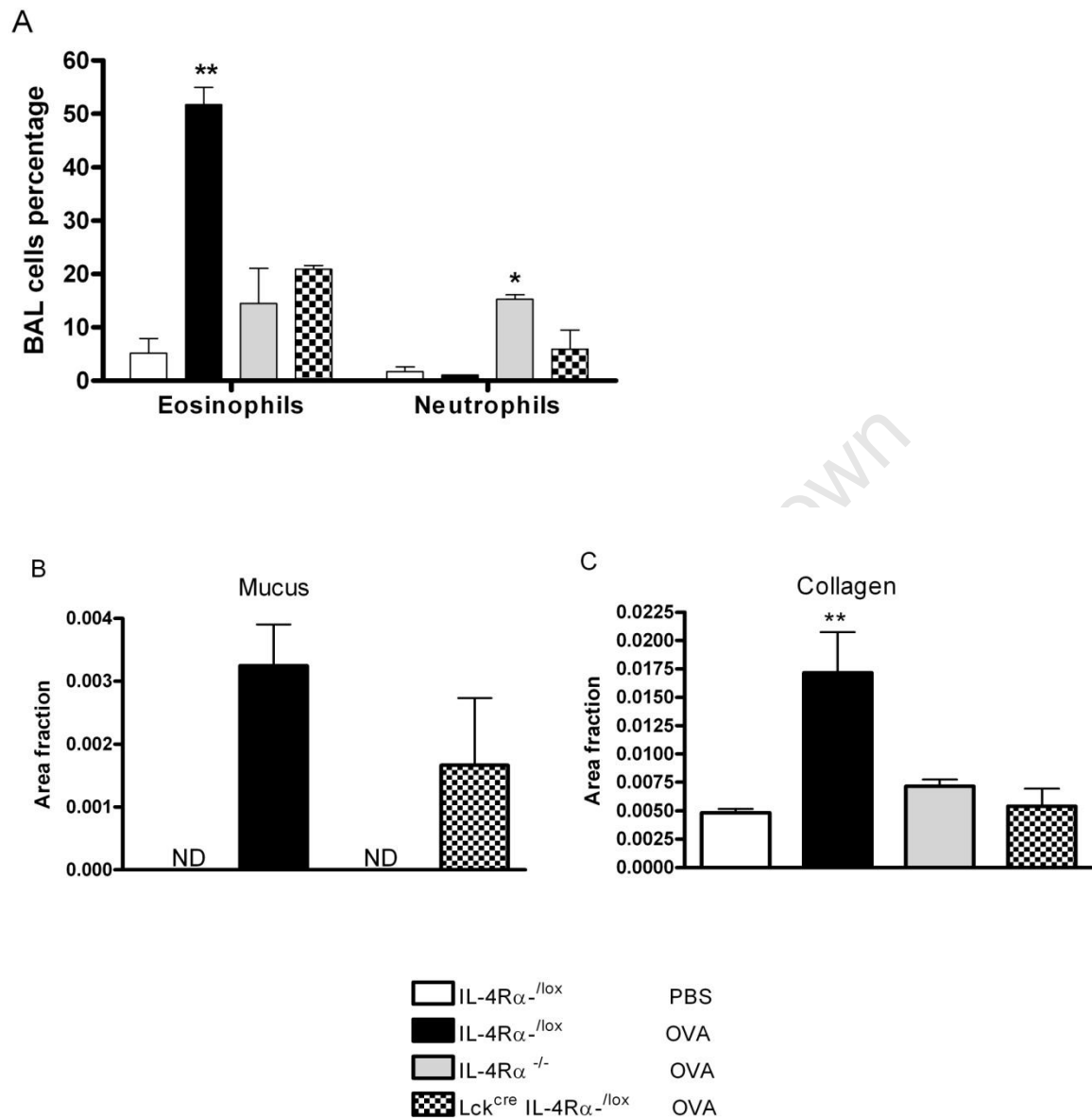
**Figure 1. Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  OVA mice are protected against AHR.** Airway Resistance (A) and elastance (B) were significantly decreased in Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  OVA mice compared to IL-4R $\alpha^{-/-}$  PBS mice and IL-4R $\alpha^{-/-}$  OVA mice. N= 10-16 mice, pooled data from three individual experiments. Significant differences between IL-4R $\alpha^{-/-}$  PBS and Lck<sup>cre</sup> IL-4R $\alpha^{-/-}$  mice are represented by stars (\*, P<0.05, \*\*, P<0.01), \*\*\*, P<0.001) and significant differences between IL-4R $\alpha^{-/-}$  OVA and Lck<sup>cre</sup> IL-4R $\alpha^{-/-}$  mice are represented by hashes (#, P<0.05, ###, P<0.001). P values are in comparison to individual responses and different concentrations of inhaled methacholine.

#### 2.4.2 Markers of allergic inflammation are reduced in $Lck^{cre}IL-4R\alpha^{-/lox}$ mice

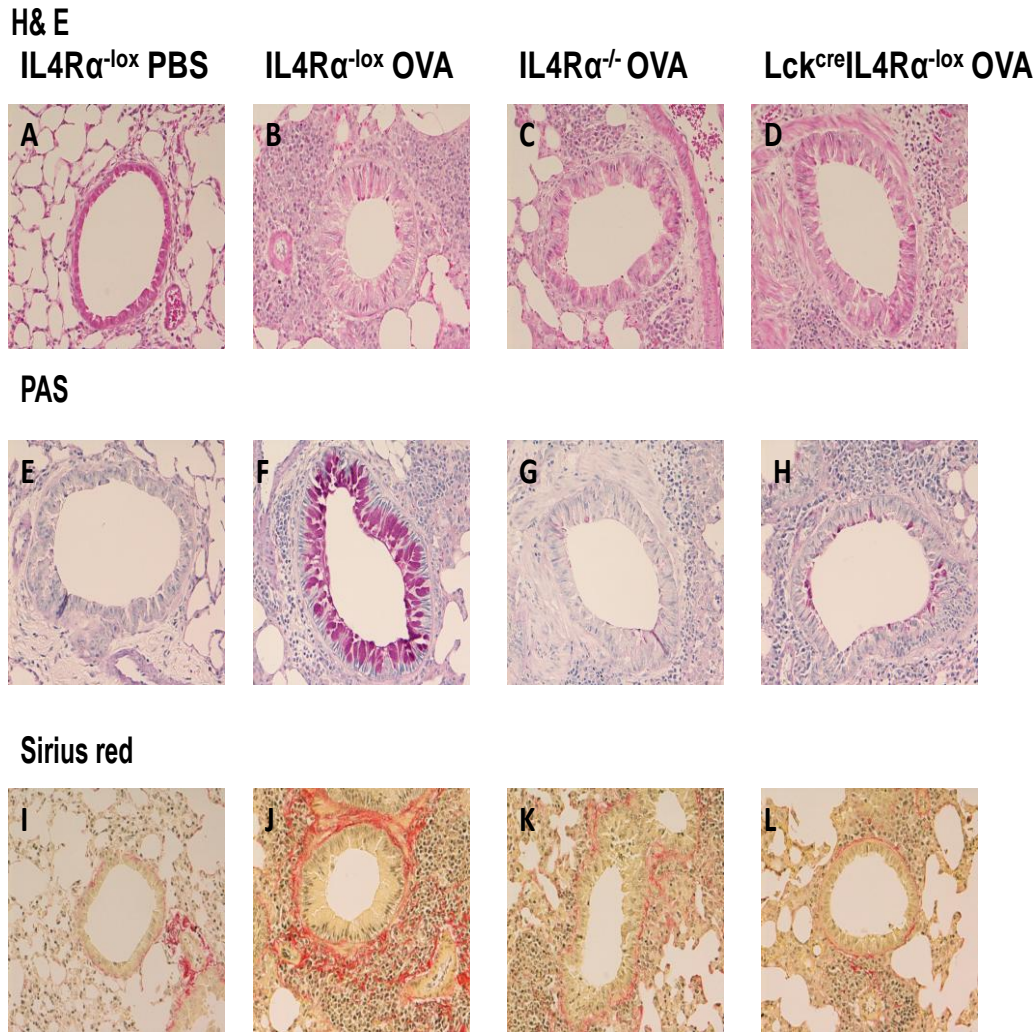
As a consequence of OVA sensitisation and challenge, inflammatory cells are recruited to the lungs. Analysis of the cellular composition of BAL fluid showed that  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice sensitised and challenged with OVA had decreased percentage of eosinophils similar to PBS controls and decreased in comparison with  $IL-4R\alpha^{-/lox}$  littermate control mice sensitised and challenged with OVA (Fig 2A). The global knockout mice  $IL-4R\alpha^{-/-}$  OVA sensitised and challenged with OVA had also decreased percentage of eosinophils compared to  $IL-4R\alpha^{-/lox}$  littermate control mice. The percentage of neutrophils in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice was decreased same as in all mice groups except for  $IL-4R\alpha^{-/-}$  mice sensitised and challenged with OVA where it was increased.  $IL-4R\alpha^{-/lox}$  OVA mice had increased mucus hypersecretion (quantified by image Analysis on NIS Elements software) compared with PBS controls, but mucus hypersecretion was abrogated in the global  $IL-4R\alpha^{-/-}$  strain (Fig 2B).

The levels of collagen deposition observed in  $Lck^{cre}IL-4R\alpha^{-/lox}$  OVA mice (Fig 3L) was decreased and similar to  $IL-4R\alpha^{-/lox}$  PBS mice (Fig 3I),  $IL-4R\alpha^{-/-}$  OVA mice (Fig 3K) compared to  $IL-4R\alpha^{-/lox}$  OVA mice (Fig 3J). Our data illustrate that  $IL-4R\alpha$  dependent  $CD4^{+}$  T cells are necessary for the development of OVA-induced allergic airway inflammation and do not appear to regulate collagen deposition.

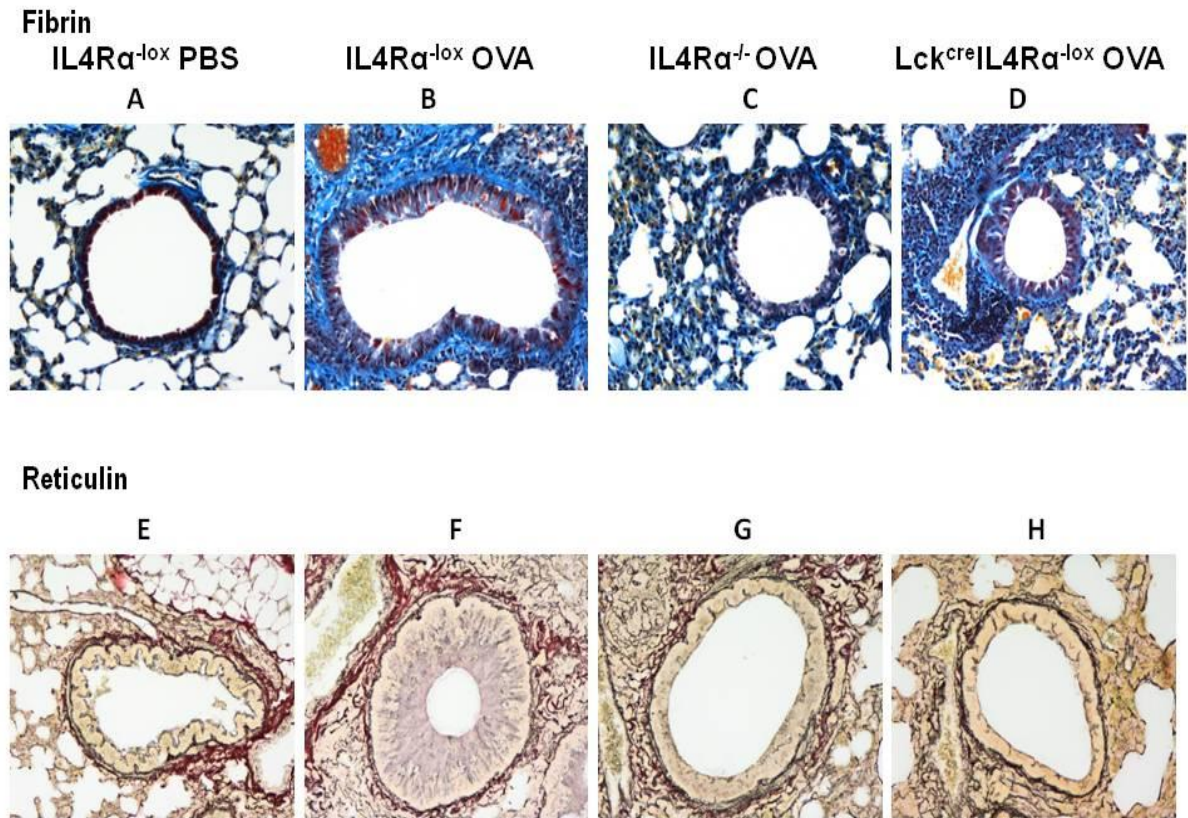




**Figure 2. Markers of lung inflammation are reduced in Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> OVA mice.** BAL fluid (A) showed a decrease of eosinophils in Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice sensitised and challenged with OVA compared to IL-4Rα<sup>-lox</sup> mice sensitised and challenged with OVA. Mucus hypersecretion (B) and collagen deposition (C) was reduced in Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice compared to IL-4Rα<sup>-lox</sup> mice sensitised and challenged with OVA mice. Significance was to IL-4Rα<sup>-lox</sup> PBS. N= 8-12, pooled data from two different experiments. (\*, P<0.05, \*\*, P<0.01)



**Figure 3. Airway infiltration of the lung tissue with inflammatory cells, goblet cell hyperplasia and collagen deposition is affected by loss of IL-4R $\alpha$  on CD4<sup>+</sup> T cells.** Lung sections from IL-4R $\alpha$ <sup>-lox</sup> mice sensitised and challenged with saline /PBS (A) and OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (B), IL-4R $\alpha$ <sup>-/-</sup> mice (C) and Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (D) were stained with H&E. Mucus hyper secretion by goblet cells were stained with PAS in the airways of PBS sensitised and challenged IL-4R $\alpha$ <sup>-lox</sup> mice (E) and OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (F), IL-4R $\alpha$ <sup>-/-</sup> mice (G) and Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (H). Collagen deposition around airways and blood vessels were illustrated by Sirius red staining in IL-4R $\alpha$ <sup>-lox</sup> mice sensitised and challenged with saline /PBS (I) and OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (J), IL-4R $\alpha$ <sup>-/-</sup> mice (K) and Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (L).



**Figure 4. Airway remodelling is affected by loss of IL-4R $\alpha$  on CD4<sup>+</sup> T cells.** Lung sections from IL-4R $\alpha$ <sup>-lox</sup> mice sensitised and challenged with saline /PBS (A) and OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (B), IL-4R $\alpha$ <sup>-/-</sup> mice (C) and Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (D) were stained with Martius scarlet blue. Increased fibrin threads (stained red) were observed in the airways of OVA sensitised and challenged IL-4R $\alpha$ <sup>-lox</sup> mice (B) and very little fibrin threads were observed in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (D). Epithelial thickness and subepithelial collagen deposition were illustrated by reticulin fibre staining. OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (F) showed loose arrays of reticular fibres (stained black) around the alveolar walls and increased collagen deposition (in red) around the airways compared to the other strains of mice.

The infiltration of inflammatory cells in the lung tissue around airways and blood vessels is a characteristic of allergic airway disease. During chronic allergic airway disease, H&E staining showed no infiltration of inflammatory cells around the airways in littermate control mice sensitised and challenged with PBS (Fig 3A). IL-4R $\alpha^{-/lox}$  mice sensitised and challenged with OVA showed severe infiltration of inflammatory cells around the airways (Fig 3B). There was a reduction in inflammation observed both in IL-4R $\alpha^{-/-}$  OVA mice (Fig 3C) and Lck<sup>cre</sup>IL-4R $\alpha^{-/lox}$  OVA mice (Fig. 3D).

Mucus hypersecretion by goblet cells is a characteristic of AAD. Histological examination of lungs stained with PAS showed no signs of mucus hypersecretion in the airways of PBS sensitised and challenged IL-4R $\alpha^{-/lox}$  mice (Fig. 3E) compared to IL-4R $\alpha^{-/lox}$  OVA mice (Fig. 3F) which showed mucus hyper secretion indicating the severity of goblet cell hyperplasia. IL-4R $\alpha^{-/-}$  OVA mice showed no mucus secretion (Fig. 3G). However Lck<sup>cre</sup>IL-4R $\alpha^{-/lox}$  OVA mice revealed little mucus secretion around the airways (Fig. 3H) compared to IL-4R $\alpha^{-/lox}$  OVA mice.

Sirius red staining showed little collagen deposition around airways and blood vessels in mice sensitised and challenged with PBS (Fig. 3I) compared to the IL-4R $\alpha^{-/lox}$  OVA mice where there was increased collagen deposition. (Fig. 3J) Both Lck<sup>cre</sup>IL-4R $\alpha^{-/lox}$  mice (Fig. 3K) and IL-4R $\alpha^{-/-}$  mice (Fig.3L) showed little collagen deposition around airways and blood vessels.

Fibrin deposition occurs as a result of inflammatory reactions in AAD. Martius Scarlet blue staining showed increased fibrin mass around the airways of OVA sensitised and challenged IL-4R $\alpha^{-/lox}$  littermate control mice (Fig. 4B) compared to IL-4R $\alpha^{-/lox}$  PBS mice (Fig. 4A) . IL-4R $\alpha^{-/-}$  OVA mice showed no fibrin threads (Fig. 4C). However Lck<sup>cre</sup>IL-4R $\alpha^{-/lox}$  OVA mice revealed few fibrin threads around the airways (Fig. 4D) compared to IL-4R $\alpha^{-/lox}$  OVA mice. In chronic AAD, airway remodelling includes structural changes such as epithelial shedding and increased subepithelial collagen deposition. Reticulin silver staining showed increased thickness of basement membrane and loose arrays of reticulin fibres in the airways of OVA sensitised and challenged IL-4R $\alpha^{-/lox}$  littermate control mice (Fig. 4F) compared to IL-4R $\alpha^{-/lox}$  PBS mice (Fig. 4E) . IL-4R $\alpha^{-/-}$  OVA and Lck<sup>cre</sup>IL-4R $\alpha^{-/lox}$  OVA mice showed thick array of reticulin fibres (Fig. 4G and 4H).

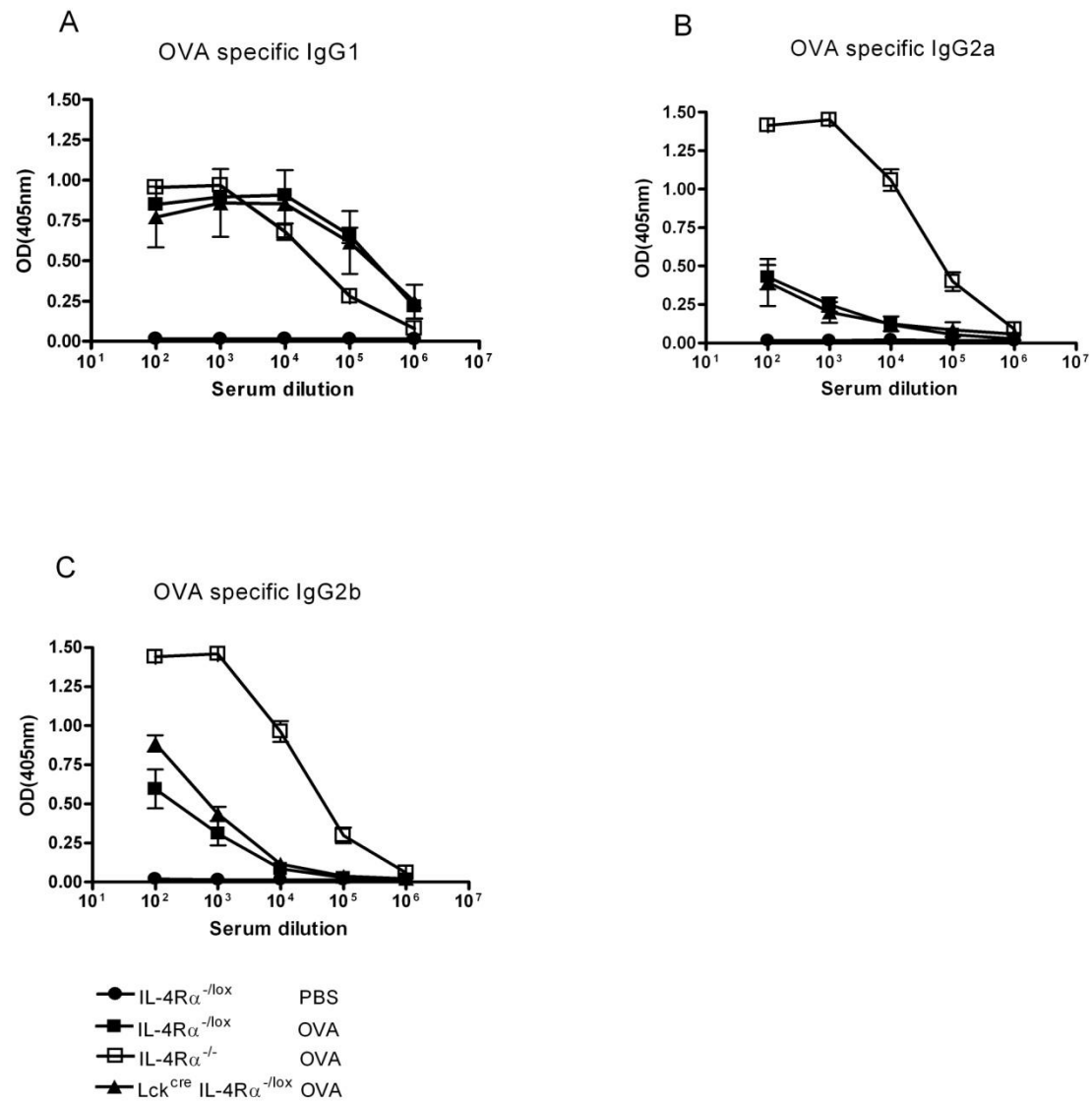
### 2.4.3 Th2 type antibodies, Th2 cytokines and Th17 responses are altered in $Lck^{cre}IL-4R\alpha^{-/lox}$ mice

In order to investigate if the antibody responses to chronic OVA sensitisation and challenge are affected by IL-4R $\alpha$  signalling on CD4<sup>+</sup>T cells, OVA specific antibody levels in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice were compared to those of  $IL-4R\alpha^{-/lox}$  and  $IL-4R\alpha^{-/-}$  mice.

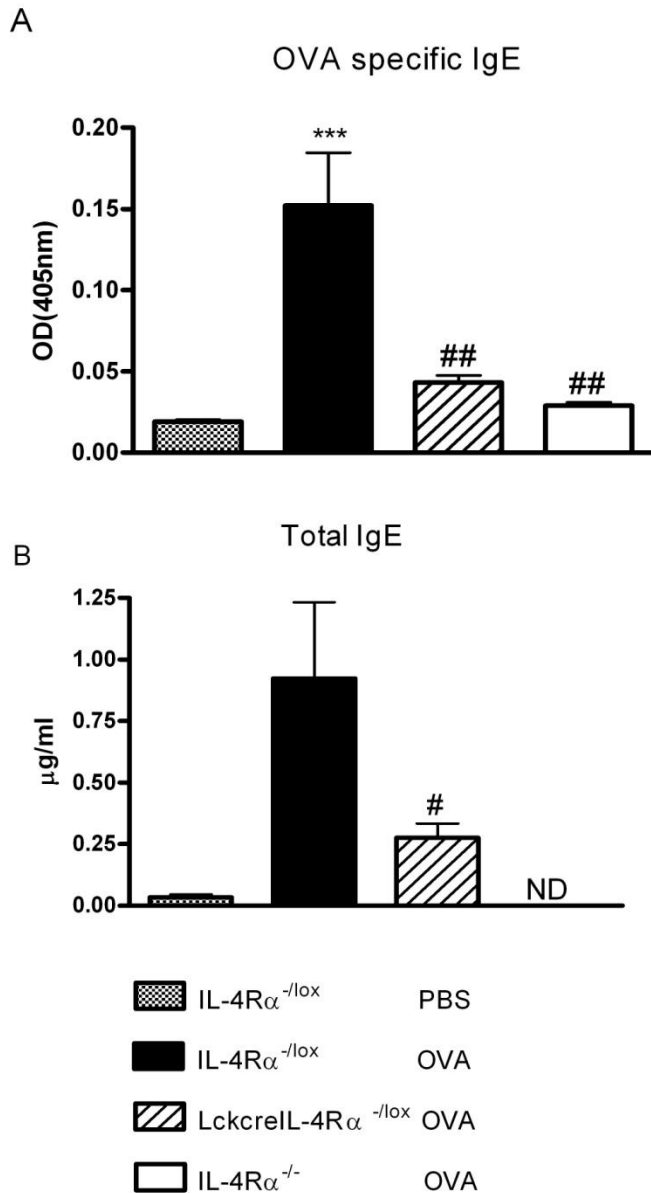
A higher serum IgG1 was observed in  $Lck^{cre}IL-4R\alpha^{-/lox}$  OVA mice compared to  $IL-4R\alpha^{-/lox}$  PBS mice and it was similar to  $IL-4R\alpha^{-/lox}$  OVA mice.  $IL-4R\alpha^{-/-}$  OVA mice also showed a higher IgG1. (Fig 5A) Lower IgG2a and IgG2b titers were observed in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice compared to  $IL-4R\alpha^{-/-}$  mice.  $IL-4R\alpha^{-/lox}$  PBS and OVA mice also showed lower IgG2a and IgG2b. As expected,  $IL-4R\alpha^{-/-}$  mice showed significantly increased levels of specific IgG2a and IgG2b antibody titers. (Fig 5B and 5C) Thus IgG responses in OVA sensitised and challenged  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice are similar to the littermate control OVA mice.

Concentration of Ovalbumin specific IgE was significantly reduced in OVA sensitised and challenged  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice compared to  $IL-4R\alpha^{-/lox}$  OVA mice. (Fig 6A) Total IgE antibody titer was also significantly reduced in  $Lck^{cre}IL-4R\alpha^{-/lox}$  OVA mice compared to littermate control mice. (Fig 6B)

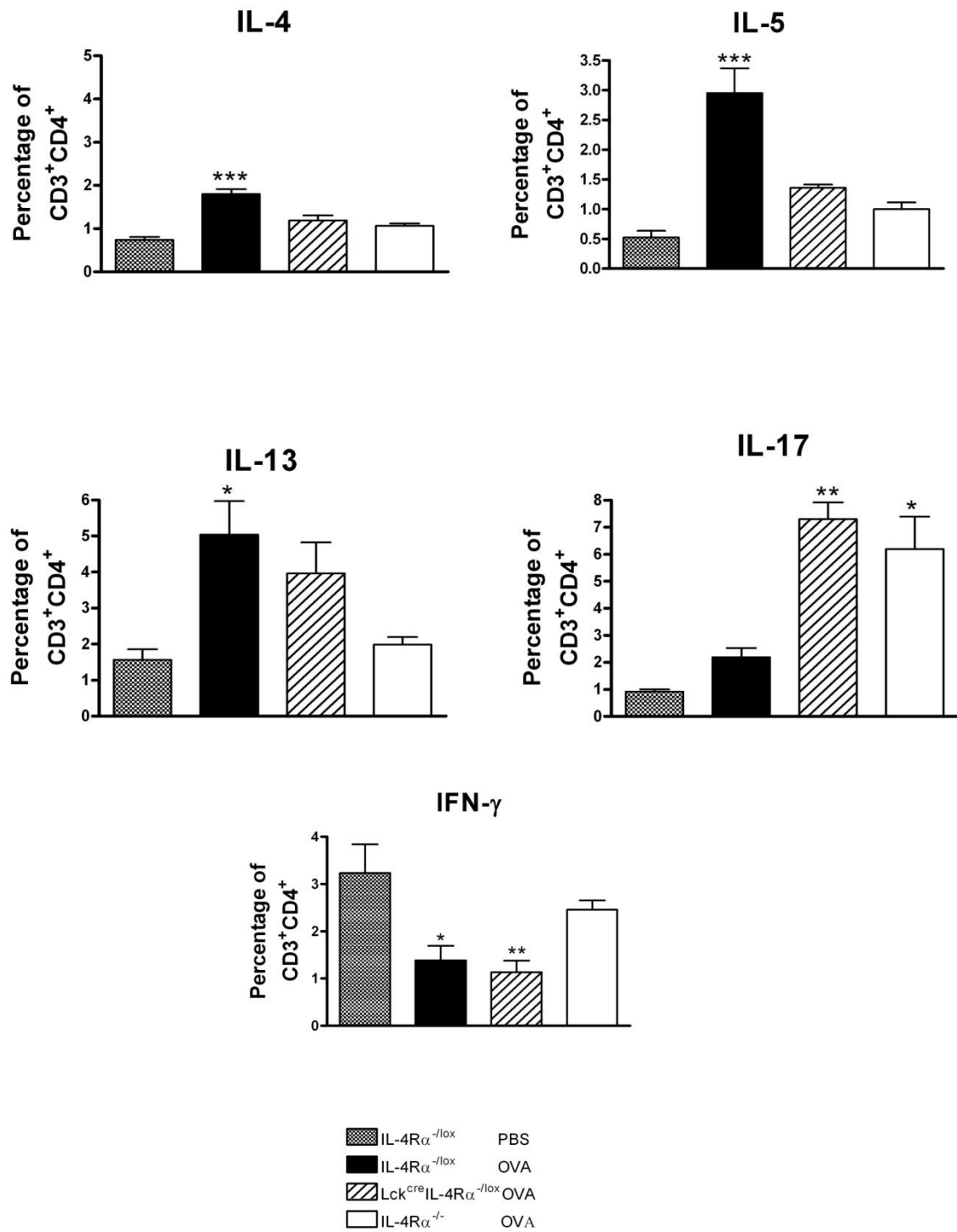
Thus our results confirm that the humoral immune response in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice is mediated by OVA specific antibody responses.



**Figure 5.** OVA specific antibody responses of Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice are similar to IL-4R $\alpha$ <sup>-lox</sup> littermate control mice and indicate a predominantly Th2-type response. OVA specific antibodies were measured by end point titration ELISA. IgG1 (A), IgG2a (B), IgG2b (C) subclasses were tested. Data represents three individual experiments (N=5 mice).



**Figure 6. OVA specific and Total IgE antibody levels were significantly reduced in  $Lck^{cre}IL-4R\alpha^{-/lox}$  mice compared to  $IL-4R\alpha^{-/lox}$  mice.** OVA specific IgE (A) and total IgE (B) antibodies were measured by ELISA. (ND Non Detectable) Significant differences between  $IL-4R\alpha^{-/lox}$  PBS and  $Lck^{cre} IL-4R\alpha^{-/lox}$  mice are represented by stars (\*\*\*,  $P<0.001$ ) and significant differences between  $IL-4R\alpha^{-/lox}$  OVA and  $Lck^{cre} IL-4R\alpha^{-/lox}$  mice are represented by hashes (#,  $P<0.05$ , ##,  $P<0.01$ ).

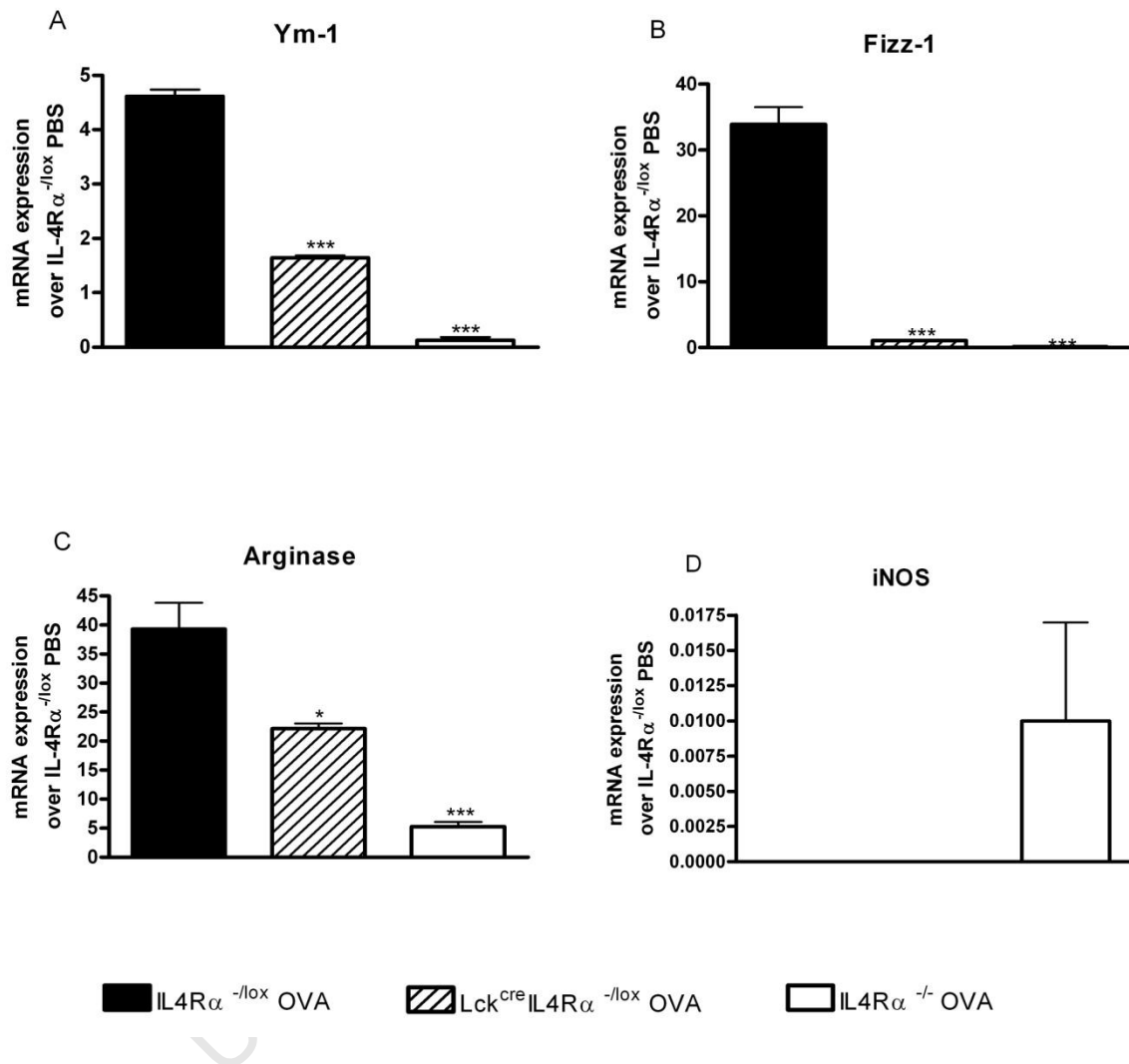


**Figure 7. Sensitisation and challenge with OVA induces a Th2 type immune response in IL-4Rα<sup>-/-</sup> and reduced in Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice.** Lung cells were incubated at 37°C for 4 hours with phorbol myristate acetate (PMA), ionomycin and monensin for intracellular cytokine staining. N=5-8 mice, pooled data from two individual experiments. Significant differences to IL-4Rα<sup>-/-</sup> mice sensitised and challenged with PBS. (\*\*, P<0.01, \*\*\* P<0.001).



The percentages of IL-4, IL-5 and IL-13 producing CD4<sup>+</sup> T cells in the lungs of Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice sensitised and challenged with OVA were reduced compared with IL-4Rα<sup>-/lox</sup> OVA littermate controls. Lungs of IL-4Rα<sup>-/lox</sup> OVA mice showed significant percentage increases of IL-4, IL-5 and IL-13 producing CD4<sup>+</sup> T cells compared with PBS littermate controls. The percentage of IL-17 producing CD4<sup>+</sup> cells was significantly increased both in IL-4Rα<sup>-/-</sup> and Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice sensitised and challenged with OVA (Fig 7). Hence our results confirm that the absence of IL-4Rα on CD4<sup>+</sup> T cells did suppress Th2 and Th17 responses in allergic airway disease.

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**Figure 8. mRNA expression in alveolar macrophages normalized with rs12 housekeeping gene.** Expression of Ym-1(A), Fizz-1(B), arginase(C) and iNOS(D) expression was decreased in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice compared to IL-4R $\alpha$ <sup>-/lox</sup> OVA mice. Data is representative of 2 experiments with triplicate pooled samples.

Alveolar macrophages from OVA sensitised and challenged mice were sorted. mRNA expression of markers for classical activation (iNOS) and alternative activation (Ym-1, FIZZ-1, and Arg-1) was quantified using RT-PCR, using the rs12 housekeeping gene for normalisation. IL-4R $\alpha$ <sup>-/-lox</sup> mice showed alternative activation of macrophages as there was increased expression of Ym-1, FIZZ-1, and arginase in comparison to saline controls. (Fig. 8 A, B, C). The expression of arginase, Ym-1, FIZZ-1 and iNOS was decreased in macrophages of Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice compared to IL-4R $\alpha$ <sup>-/-lox</sup> mice showing that in the absence of IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells, there is reduced classical and alternative activation of macrophages.

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## 2.5 Discussion

Allergic asthma is characterised by Th2 inflammatory responses in the lung orchestrated by CD4<sup>+</sup> T cells.<sup>41</sup> Studies exploring T cell therapies have been conducted over two decades ago but without any convincing results.<sup>42</sup> The blocking of T cells in asthmatic patients showed some improvement in pathology of asthmatics.<sup>43</sup> But the use of anti-CD4 monoclonal antibody did not reduce the symptoms of asthma.<sup>44, 45</sup> Th2 cytokine blocking Therapies involving anti-cytokine agents were introduced to curb inflammation in asthma. Studies conducted in human, aimed to target the main Th2 cytokines including IL-4, IL-5, IL-9 and IL-13. IL-4 blockade in mouse models of asthma showed abrogated pathology in asthma.<sup>46</sup> Some promising evidence has been shown by the administration of solubilised IL-4 receptor fragment in treating asthma.<sup>47</sup> Clinical trials have shown the lack of efficacy of the soluble IL-4 receptor and anti-IL-4 humanised monoclonal antibody.<sup>48, 49</sup> The key role of IL-5 and use of IL-5 antibody in asthma pathology have been demonstrated by studies conducted in murine and guinea pig models.<sup>50-53</sup> Human studies involving administration of human IL-5 antibody also failed to show prevention of asthmatic symptoms.<sup>54-56</sup> In mouse models of asthma, blocking IL-9 by antagonists reduced allergen-induced airway inflammation and AHR. The use of anti-IL-13 monoclonal antibodies and soluble IL-13 receptor showed reduced inflammation in asthma.<sup>57-59</sup> *In vitro* and humanised animal studies using IL-13-neutralizing antibody showed promising results and its clinical development is underway.<sup>60</sup>

Lung biopsies and respiratory secretions from asthmatic patients have demonstrated that CD4<sup>+</sup> T cell infiltration around the airways and eosinophil recruitment are key events of the inflammatory process contributing to disease severity.<sup>61-63</sup> Murine models have demonstrated that CD4<sup>+</sup> T cells and IL-5 secretion by these cells, in conjunction with eosinophil recruitment at inflammatory sites, contribute to the pathophysiology of allergic airways inflammation.<sup>14</sup> Other studies using chronic mouse models have shown that even though CD4<sup>+</sup> T cells contribute to eosinophilic lung inflammation, they are not required for subsequent airway remodeling.<sup>14</sup> In murine T cells, IL-4 mediates multiple effects. In addition these cells do not respond to IL-1 which plays a role in immune and inflammatory responses.<sup>64</sup> Therefore, the model of CD4<sup>+</sup> T cell-specific IL-4R $\alpha$ -deficient mice (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) allows the investigation of the role of IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells while maintaining IL-4/IL-13-mediated functions on non-CD4<sup>+</sup> T cells.

In a recent study in our laboratory (unpublished), it has been demonstrated that IL-4R $\alpha$  deletion on CD4<sup>+</sup> T cells in an acute model of AAD promotes disease progression. There has been no studies documented that defines the direct role of IL-4R $\alpha$  on CD4<sup>+</sup> T cells in chronic allergic airway disease and airway remodelling. In order to investigate the role of IL-4R $\alpha$  on CD4<sup>+</sup> T cells in a long-term model of murine AAD, the present study utilised previously characterized and published Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice.<sup>26-28</sup> These mice which have impaired IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells were sensitized and challenged with ovalbumin.

Our data in the present study indicates that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice are significantly protected from AAD in comparison to littermate control mice sensitised and challenged with OVA (IL-4R $\alpha$ <sup>-lox</sup>). It is observed that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice demonstrated decreased disease pathology (although not significant) in airway inflammation, mucus hypersecretion, collagen deposition and airway remodelling compared to OVA littermate control mice (IL-4R $\alpha$ <sup>-lox</sup>). In addition, we observed a significant decrease in AHR in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice when compared to OVA littermate control mice (IL-4R $\alpha$ <sup>-lox</sup>). Decreased airway remodelling in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice was confirmed by histology for fibrin and reticular fibres. Markers of AAMs such as arginase, Ym-1, and Fizz-1 were impaired in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice during OVA-induced AAD compared to OVA littermate controls (IL-4R $\alpha$ <sup>-lox</sup>). This observation could probably be a consequence of reduced Th2 cytokines and other factors produced by T cells. Together these data show that IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells play an essential role in the pathogenesis of chronic murine AAD. However, it has been demonstrated that CD4 independent mechanisms can also support recruitment and maintenance of inflammatory cells. Chemokines and cytokines secreted by airway epithelium, mast cells and eosinophils can also contribute to the inflammatory process.<sup>65-68</sup> Although it has been reported that CD4<sup>+</sup> T cells are required at later stages in establishing AAD, our data clearly demonstrate that IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells contribute significantly to the pathology of chronic AAD.

Our findings suggest two possibilities. Firstly, IL-4 dependent Th2 is important in chronic AAD and secondly, IL-4 inhibits CD4<sup>+</sup> T cell mechanisms that downregulate chronic AAD. The latter may be more likely, as Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice still have IL-13 producing CD4<sup>+</sup> T cells, albeit at decreased levels compared to littermate controls (IL-4R $\alpha$ <sup>-lox</sup>). Unpublished data from our group found that IL-13 signalling can occur via IL-13R $\alpha$ 2 which is known to be a decoy receptor.

The role of IL-17 in the pathogenesis of asthma has been studied. It has been established that IL-17, through the release of proinflammatory mediators contribute to the inflammation in asthmatics.<sup>69</sup> Increased levels of IL-17 were observed in blood and airways of asthmatic patients.<sup>70, 71</sup> Human studies have shown the proliferation and increased airway smooth muscle (ASM) mass by Th17 cytokines indicating their role in airway remodelling.<sup>72, 73</sup> IL-17F may have a crucial role in allergic airway inflammation, and have important therapeutic implications in asthma. Importantly, we determined that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice had increased IL-17 and IFN- $\gamma$  production by CD4<sup>+</sup> T cells (from lungs) throughout the course of the model. While IL-17 has been shown to induce neutrophil-dependent airway hyperreactivity, it was also found to down-regulate established allergic airway inflammation and reduce airway hyperreactivity.<sup>74-77</sup> From a previous publication, it was shown that IL-17 produced by CD4<sup>+</sup> T cells was important in downregulating airway inflammation in mice in a model of bleomycin-induced lung injury<sup>78</sup>. However, human studies have found an association between IL-17 and severe asthma, which is often neutrophilic and corticosteroid resistant.<sup>79, 80</sup> In addition, Th17 driven allergic airway inflammation was found to be steroid resistant *in vivo*.<sup>81</sup>

The results presented here demonstrates the importance of IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells and confirms that it is detrimental in chronic murine model of AAD. Further studies are required to understand the mechanism by which IL-4R $\alpha$  on CD4<sup>+</sup> T cells contribute to the pathology of AAD. Thus IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells plays an essential role in murine AAD and plays a key role in airway remodelling.

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## **CHAPTER 3**

**IL-4R $\alpha$  expression on Macrophages/Neutrophils is  
not required for chronic Allergic Airway Disease  
(AAD) in mice**

### **3 IL-4R $\alpha$ expression on Macrophages/Neutrophils is not required for chronic Allergic Airway Disease (AAD) in mice**

#### **3.1 Summary**

##### **Background**

Alternative activation of macrophages is induced by the Th2 cytokines IL-4 and IL-13 through IL-4R $\alpha$ . Markers of alternatively activated macrophages (AAMs) are upregulated in asthmatic lungs and mice with allergic airway disease (AAD), the role of IL-4R $\alpha$  activated AAMs in the development of AAD is not well defined. By using transgenic LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice deficient for IL-4R $\alpha$  in macrophages and neutrophils, the *in vivo* effects of impaired IL-4R $\alpha$  signalling in macrophages and the role of AAMs on the outcome of AAD were investigated.

##### **Objective**

The aim of this study is to investigate the chronic effects of AAD and the role of IL-4R $\alpha$  activated AAMs in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice.

##### **Methods**

AAD was introduced in mice by repeated subcutaneous sensitisation with 20  $\mu$ g ovalbumin (OVA) adsorbed to 0.65% alum on days 0, 7, 14, and 21. On days 27 and 29 and then twice weekly for 4 weeks, mice were allergen challenged intranasally with 20  $\mu$ g OVA in 50  $\mu$ L PBS under anaesthesia. Mice were investigated for the presence of airway hyperresponsiveness (AHR), markers of allergic inflammation, allergen specific Th2 type antibody and cytokine production, arginase activity and nitric Oxide production.

##### **Results**

Airway hyperreactivity, Th2 responses, mucus hypersecretion, eosinophil infiltration, and collagen deposition were not reduced in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice but AAM expression (Ym-1 and arginase) was decreased in macrophages of LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice.

## Conclusion

These findings demonstrate that there is no reduction in AAD in mice in the absence of IL-4R $\alpha$  dependent AAMs, suggesting that AAMs are not critical for the outcome of allergic airway disease.

## 3.2 Introduction

Alternatively activated macrophages are associated with murine AAD and human asthma<sup>1, 2</sup>. IL-4 and IL-13 induce alternative activation of macrophages through IL-4R $\alpha$ . The role of IL-4R $\alpha$  dependent AAMs in the pathology of AAD was investigated in this study by using LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice.

Monocytes derived from pluripotent hematopoietic stem cells in the bone marrow continually migrate into the tissues where they mature into macrophages. Macrophages, the most abundant immune cells in the lung reside in the alveolar spaces of the lung contributing to the innate immune defense in the airways by expressing a variety of pattern recognition receptors like mannose receptors or  $\beta$ -glucan receptors<sup>3,4</sup>. The two main subsets of lung macrophages are alveolar macrophages (AM) and interstitial macrophages (IM) which are phenotypically and functionally distinct. IMs express high levels of MHC II, show greater efficiency in stimulating T cell proliferation *in vitro* even though have lower phagocytic potential. Thus AMs and IMs have complementary immunosuppressive effects with differential DC function and pulmonary immune responses.<sup>5</sup> Upon an inflammatory stimulus, monocyte derived macrophages are recruited to the lung and *in vivo* studies have demonstrated that both resident and recruited macrophages can alternatively activate and be driven to proliferate by a Th2 environment.<sup>6</sup> Macrophages proliferate locally upon stimulation and are efficient in phagocytosing debris and microbes by production of inflammatory cytokines.<sup>3,6,7</sup> and also in repairing tissues by release of trophic substances.<sup>3,7,8</sup>

The activation of macrophages is important for their functioning<sup>7, 9</sup> and exhibit multiple phenotypes depending on the cytokine environment and disease context. The two conventional macrophage types identified were: classically activated macrophages (CAM) and alternatively activated macrophages (AAM).<sup>10</sup> However another two novel macrophage

phenotypes called oxidized phospholipid-treated murine macrophages (Mox)<sup>11</sup> and Tumour-associated macrophages (TAMs) has also been reported.<sup>12</sup>

Th1-associated classically activated macrophages (CAM) are pro-inflammatory, activated by Th-1 cytokine IFN- $\gamma$ , secrete TNF- $\alpha$  and involved in the upregulation of inducible nitric oxide synthase (iNOS), resulting in nitric oxide (NO) production by hydrolysis of L-arginine. CAMs mediate defence of the host by efficient bacterial killing and promotes extracellular matrix (ECM) degradation and apoptosis causing tissue damage.<sup>13</sup> In contrast to the classically activated macrophages, the Th2-associated alternatively activated macrophage (AAMs) secretes ECM components such as trans-glutaminase, fibronectin and osteopontin. These macrophages got pro-fibrotic effects by eliciting immunity against multicellular pathogens, are anti-inflammatory and regulate wound healing.<sup>6, 14</sup> Mox macrophages show decreased phagocytotic and chemotactic capacity whereas TAMs suppress antitumour immunity.

IL-4/IL-13-activated alternative macrophages (AAMs) are characterised by IL-4R $\alpha$ -driven increased arginase-1 expression,<sup>7,9,15,16</sup> induction of chitinase and FIZZ family members (ChAFFs)<sup>17</sup> and chitinase-like molecules (e.g. Ym-1),<sup>17-19</sup> along with other biomarkers such as peroxisome proliferator-activated receptor  $\gamma$  (PPAR  $\gamma$ ) and mannose receptor (MR). The enzyme Arginase-1, a marker gene for alternative activation<sup>7</sup> uses L-arginine as a substrate to produce L-ornithine and urea<sup>20</sup>. These products are then used in the biosynthesis of proline and polyamines<sup>9, 20</sup>. AAMs produce increased levels of arginase-1 thereby suppressing NO production. Prolines are precursors of collagen and are associated with the construction of ECM. Polyamines are molecules associated with cell proliferation and are important in the generation of connective tissue, smooth muscle, and mucus.<sup>7,9,15</sup> Thus the production of arginases constitute an array of anti-inflammatory, tissue repair and pro-fibrotic-associated responses.<sup>21</sup> L-arginine is the same substrate used by the CAM-associated iNOS and thus these two enzymes are representative of the M1/M2 dichotomy<sup>20</sup>.

IL-4R $\alpha$ -responsive macrophages which are capable of developing into the alternatively activated phenotype have been associated with being protective against a range of pathology<sup>15, 22-24</sup>. Alternatively activated macrophages, or M2 macrophages, have been associated with the control of inflammation<sup>22,23</sup>, down-regulation of Th2 responses, enhanced wound healing

<sup>24</sup>, and tissue damage <sup>25</sup> in the lung. Additionally, AAMs have been shown to control viral-induced lung damage in an IL-4R $\alpha$  dependent way. <sup>26</sup>

It has been hypothesized that the pathogenesis of AAD is contributed by AAMs by increased levels of arginase production and by synthesising proline and polyamines, the molecules involved mainly in the generation of connective tissue, smooth muscle, and mucus. Studies in both mouse models and human studies have demonstrated an upregulation in markers of AAMs such as arginase, found in inflammatory zone 1 (FIZZ-1), chitinases, and chitinase-like proteins in the allergic lung. <sup>2, 27, 28</sup> AAMs were thought to play an important role in asthma by inhibiting the production of NO which is necessary for the regulation of airway and vascular tone. It also promotes the synthesis of collagen and other connective tissues <sup>2,22,29,30</sup> increased secretion of mucus <sup>29</sup> and recruitment of eosinophils. <sup>22, 31</sup>

However, other studies have demonstrated that AAMs may have regulatory or suppressive properties. Infection with nematodes such as *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* in mice showed that induction of AAM by memory Th2 cells mediated a protective immune response against the parasite <sup>32</sup> leading to the induction of AAM in the lung. <sup>25,33</sup> Infection with other nematodes such as that of *Brugia malayi*, have shown that AAMs suppress CD4<sup>+</sup> T-cell proliferation *in vitro* <sup>34</sup> and IL-4-dependent alternative activation of macrophages can occur without adaptive immunity, contributing to wound healing <sup>22</sup>. AAMs are also involved in downregulating a harmful Th1 immune response <sup>24</sup> when mice were infected with *Schistosoma mansoni*. Chronic pulmonary infection of *Cryptococcus neoformans* in mice was accompanied with induction of AAM leading to the development of symptoms similar to allergic asthma. <sup>35,36</sup> AAMs were also involved in other diseases such as filarial infection, <sup>37</sup> arthritis, <sup>38</sup> experimental autoimmune encephalomyelitis, <sup>39</sup> lung fibrosis <sup>40,41</sup> and they can also downregulate T-cell proliferation. <sup>6,37,42-44</sup>

Mouse models of AAD have shown induction of AAM in the lung after allergen challenge. The expression of the chitinases Ym1 and 2 in alveolar macrophages and upregulation of arginase-1<sup>2</sup> was dependent on IL-4R $\alpha$  signalling in an ovalbumin model of allergic asthma. <sup>30,45,46</sup> Arginase 1 inhibition by RNA interference attenuated IL-13 induced allergic symptoms. <sup>47</sup> In addition, IL-13R $\alpha$ 1 signalling is not necessary to induce AAM differentiation in a mouse model of AAD suggesting that IL-4R $\alpha$  signalling might be sufficient. <sup>48</sup> In atopic

asthma patients, the numbers of AAM were increased in comparison to healthy controls <sup>1</sup> and high numbers of arginase expressing macrophages were also found in the bronchoalveolar lavage (BAL).<sup>2</sup> An increase in the number of airway macrophages was found both in patients with asthma <sup>49-51,54</sup> and animal models of AAD <sup>52-53</sup>. On the contrary, other studies in rats and mice showed suppression of allergic symptoms by the modulation and phagocytosis function of alveolar macrophages and allergen-loaded macrophages. <sup>55-58</sup> However this suggests that macrophages may play an important role in allergic inflammation of the lung but the exact role of AAM in AAD still needs to be established.

Therefore, we aimed to test the hypothesis that IL-4R $\alpha$  dependent AAMs contribute to ovalbumin induced AAD by using LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice, which have abrogated IL-4R $\alpha$  signalling on macrophages and neutrophils. Our results demonstrate no reduction in allergic airway disease in the absence of IL-4R $\alpha$  dependent AAMs, suggesting that AAMs may not be as important in allergic airway disease as is commonly suggested.

### 3.3 Materials and Methods

#### Mice

Eight- to ten week-old  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$ <sup>7</sup>,  $\text{IL-4R}\alpha^{-/\text{lox}}$ , and  $\text{IL-4R}\alpha^{-/-}$  female mice<sup>27</sup> on a BALB/c background were housed in independently ventilated cages under specific pathogen-free conditions in the University of Cape Town Animal Facility. Animal procedures were approved by the University of Cape Town Animal Ethics Committee. (Permit Number: 008/016)

#### Generation of IL-4R $\alpha$ knockout mice

$\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice<sup>24</sup> were generated by mating floxed  $\text{IL-4R}\alpha^{\text{lox/lox}}$  mice with transgenic  $\text{LysM}^{\text{cre}}$  mice.<sup>59</sup> These mice were then back-crossed to the BALB/c background for 9 generations prior to crossing with global IL-4R $\alpha$  knockout ( $\text{IL-4R}\alpha^{-/-}$ ) mice<sup>60</sup> to finally obtain  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  and  $\text{iLck}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  BALB/c mice.  $\text{IL-4R}\alpha^{-/\text{lox}}$  littermates and  $\text{IL-4R}\alpha^{-/-}$  mice were used as controls in all experiments.

#### Genotyping of IL-4R $\alpha$ knockout mice

Mice were genotyped via PCR to determine what IL-4R $\alpha$  allele was present and the PCR conditions were as follows: 94°C/30 s; 57°C/30 s, 72°C/1 min for 40 cycles on an MJ thermocycler (Biozym, Hessisch, Oldendorf, Germany).

Primers for PCR screening of the mice

#### Lys M Cre Transgene allele

M lys1: 5'- CTT GGG CTG CCA GAA TTT CTC -3'  
Cre Antisense: 5'- CCC AGA AAT GCC AGA TTA CG -3'

#### IL-4R $\alpha$ Wildtype allele

Exon 7 forward1: 5'-TGA CCT ACA AGG AAC CCA GGC-3'  
Exon 8 reverse: 5'-CTC GGC GCA CTG ACC CAT CT-3'

#### IL-4R $\alpha$ Deleted allele

IL-4R $\alpha$  KO P1: 5' - GGC TGC TGA CCT GGA ATA ACC - 3'  
IL-4R $\alpha$  KO P2: 5' - CCT TTG AGA ACT GCG GGC T - 3'

#### Conditional IL-4R $\alpha$ LoxP allele

IL-4R $\alpha$  KO P1: 5' - GGC TGC TGA CCT GGA ATA ACC - 3'  
IL-4R Intron 6-Rev : 5' – GTT TCC TCC TAC CGC TGA TT – 3'

### **Sensitisation and challenge of mice**

Mice were sensitized subcutaneously with 20 µg ovalbumin (OVA) adsorbed to 0.65% alum on days 0, 7, 14, and 21. On days 27 and 29 and then twice weekly for 4 weeks, mice were administered intranasal challenges with 20 µg OVA in 50 µL PBS under anaesthesia.<sup>61</sup>

### **Measurement of Airway Hyperreactivity**

Airway resistance and elastance of the whole respiratory system (airways, lung chest wall) after challenge with increasing doses of acetyl-b-methylcholine (methacholine, Sigma-Aldrich, Aston Manor, South Africa) were determined by forced oscillation measurements as described<sup>62</sup> with a Flexivent system (SCIREQ, Montreal, Canada) by using the singlecompartment (“snapshot”) perturbation. Differences in the dose-response curves were analyzed by repeated-measures ANOVA. Only mice with acceptable measurements for all doses (coefficient of determination >0.95) were included in the analysis.

### **Flow cytometry**

Bronchoalveolar lavage (BAL) cells were obtained by flushing the lungs of sacrificed mice once with 1 mL PBS/1% FCS.

Single-cell suspensions were prepared from lungs after digestion for 1 hour at 37°C in Iscove’s Modified Dulbecco’s Medium (IMDM) (Invitrogen, Carlsbad, Calif) containing 13 µg/mL DNase I (Roche, Randburg, South Africa) and 50 U/mL collagenase IV (Sigma-Aldrich, Aston Manor, South Africa).

For intracellular cytokine staining, lung cells were incubated at 37°C for 4 hours with phorbol myristate acetate (Sigma-Aldrich) (50 ng/mL), ionomycin (Sigma-Aldrich) (250 ng/mL), and monensin (Sigma-Aldrich) (200 mM in IMDM/10% FCS). Cells were stained with CD3-fluorescein isothiocyanate (clone 145-2C11) and CD4-PerCP (clone L3T4) (BD Bioscience), and intracellular cytokines were stained with anti-IL-4 (clone 11B11), anti-IL-5 (clone TRFK5), anti-IFN-γ (clone XMG 1.2), anti-IL-17 (clone TC11-18H10) (BD Bioscience), anti-IL-13 (clone eBio 13A) (eBioscience, San Diego, Calif), and rat anti-mouse IgG1 isotype control (clone A85-1) (BD Bioscience) (all PE-labeled). Cells were acquired on a FACSCalibur machine (BD Immunocytometry Systems, San Jose, Calif), and data were analyzed by using Flowjo software (Treestar, Ashland, Ore).



For flow cytometry, the following antibodies were used: SiglecF-PE (clone E50-2440), CD11c-APC (clone HL3), CD4-PerCP (clone L3T4), with streptavidin (all BD Bioscience, Erembodegem, Belgium), CD3 FITC fluorescein isothiocyanate (clone 145-2C11) and GR-1-fluorescein isothiocyanate (clone RB68C5).

### **Sorting of alveolar macrophages**

Alveolar macrophages were sorted as CD11c<sup>+</sup>SiglecF<sup>+</sup> cells from the pooled lungs of OVA-sensitized and -challenged mice. Cells were sorted on a FACSVantage flow cytometer (BD Immunocytometry Systems, San Jose, Calif). Purity was determined by flow cytometry and checked by cyto-spin and staining with the Rapidiff Stain set (Clinical Diagnostics CC, Southdale, South Africa) and was at least 99%.

### **Arginase assay**

Alveolar macrophages sorted from the lungs of OVA-sensitized and -challenged IL-4R $\alpha$ <sup>-lox</sup> and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice were plated in triplicates in 96-well plates at 2x10<sup>4</sup> cells per well in IMDM (containing 10% FCS, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin) in the presence or absence of 100 ng/mL LPS (Sigma-Aldrich). Culture plates were then incubated at 37 °C for 72 hours and following stimulation, cells were washed repeatedly with 1X PBS and lysed with 0.1% TritonX-100. Lysates were incubated with 50  $\mu$ l of 10 mM MnCl<sub>2</sub> and 50 mM Tris HCl (pH 7.5) to activate the enzyme for 10 minutes at 55°C. 25  $\mu$ l of the lysate solution was then transferred to 1.5 ml eppendorf tubes with 25  $\mu$ l 500mM L-Arginine (pH 9.7) and incubated for 1 hour at 37°C. The reaction was then stopped using 400  $\mu$ l of acid mixture (H<sub>2</sub>SO<sub>4</sub>:H<sub>3</sub>PO<sub>4</sub>:H<sub>2</sub>O at a ratio of 1:3:7). Thereafter, the colorimetric reaction was initialised by adding 25  $\mu$ l of 9%  $\alpha$ -Isonitrosopropiophenone (ISPF) and incubated at 100°C for 45 minutes, followed by 10 minutes in the dark (at room temperature). 200  $\mu$ l aliquots were added and serially diluted, three-fold, in ddH<sub>2</sub>O on a 96-well plate (Nunc Maxisorb) and read at 540 nm on a VersaMax microplate reader. Controls had ddH<sub>2</sub>O instead of L-Arginine and blanks had ddH<sub>2</sub>O added instead of cell lysates. Standards consisting of doubling dilutions of 1000  $\mu$ g/ml urea were treated under identical assay conditions. Arginase activity was calculated from the standard curve using urea concentrations ( $\mu$ g/ml).

### **Nitric oxide (NO) measurements**

Alveolar macrophages sorted from the lungs of OVA-sensitized and -challenged IL-4R $\alpha$ <sup>-/-lox</sup> and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice were plated in triplicates in 96-well plates at  $2 \times 10^5$  cells per well in IMDM (containing 10% FCS, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin) in the presence or absence of 100 ng/mL LPS (Sigma-Aldrich). Cell culture supernatants were analysed for the production of NO using the Griess reaction assay, which measures the concentration of nitrite, a stable liquid product from the reaction of NO with O<sub>2</sub>. Supernatant samples of re-stimulated lung cells were collected after 72 hours of incubation at 37°C and stored at -20°C until analysis. Supernatant samples were serially diluted three-fold and standards (1mM NaNO<sub>2</sub> solution) were serially diluted two-fold in IMDM (supplemented with 10% FCS, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin) in a total volume of 50  $\mu$ l per well in a flat bottomed 96-well plate (Nunc Maxisorb). 25  $\mu$ l of Griess Reagent 1 (1% sulfanilamide in 2.5% phosphoric acid) and then 25  $\mu$ l of Griess Reagent 2 (0.1% naphthyl-ethylene-diamine in 2.5 % phosphoric acid) were sequentially added to each well. The plate was incubated at room temperature for 5 minutes to allow the reaction to develop. The colorimetric reactions were read at 540 nm and the reference wavelength at 690 nm using a VersaMax microplate reader. The absorbance reading of the samples (in OD units) was correlated to the nitrite concentration (in mM) as calculated by the standard curve.

### **RNA extraction, cDNA synthesis and Quantitative RT-PCR**

Total RNA was extracted from sorted alveolar macrophages of >98% purity by using Tri reagent (Applied Biosystems, Carlsbad, Calif) according to the manufacturer's protocol and purified with RNeasy Microprep kit (Qiagen Hilden, Germany). Sorted alveolar macrophages were lysed in 500  $\mu$ l TriReagent® (Molecular Research, USA) and stored at -80 °C. To extract the RNA, thawed samples were incubated at room temperature for 5 min to ensure complete dissociation of nucleoproteins. To each sample, 0.1 ml of chloroform was added and samples were vortexed for 15 seconds and incubated at room temperature for a further 15 min prior to centrifugation at 12000 rpm for 15 min at 4 °C. The RNA contained in the upper aqueous layer was purified using the miRNeasy Mini Kit (Cat. No. 217004, Qiagen, USA), RNeasy MinElute Cleanup Kit (Cat. No. 74204, Qiagen, USA) and the RNase-Free DNase Set (Cat. No. 79254, Qiagen, USA). RNA quantity was determined using a Nanodrop ND1000 (Thermo Scientific, USA) and the integrity was determined using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513, Agilent Technologies, USA) and the Agilent Small RNA

Kit (Cat. No. 5067-1548, Agilent Technologies, USA) on an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The Agilent assays were performed by the Centre for Proteomic and Genomic Research, Cape Town.

cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche). Real-time quantitative PCR was performed on cDNA samples to determine gene expression levels of marker genes by using Lightcycler FastStart DNA MasterPLUS SYBR Green I reaction mix (Roche) on a lightcycler 480 II (Roche). Each reaction contained SYBR® Green Master Mix (Cat. No. 04913850001, Roche, Germany), primers and cDNA template. Reactions were prepared in 96- or 384-well plates (Roche, Germany) using a QIAgility robot (Qiagen, USA) and PCRs were performed in a LightCycler® 480 (Roche, Germany). Primers for inducible nitric oxide synthase (iNOS), arginase, Ym-1, and FIZZ-1 have been previously described and are listed in table 3.1<sup>66</sup> Gene expression levels were normalized according to the expression of the housekeeping genes ribosomal rs12.

**Table 3.1: RT-PCR primers and PCR conditions**

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing Temperature (°C)	Extension time (s)	Fluorescence acquisition temperature (°C)
<b>Ym-1</b>	GGGCATACCT TTATCCTGAG	CCACTGAAGT CATCCATGTC	60	12	80
<b>Fizz-1</b>	TCCCAGTGAA TACTGATGAG A	CCACTCTGGA TCTCCCAAGA	60	9	80
<b>Arg1</b>	CAGAAGAATG GAAGAGTCAG	CAGATATGCA GGGAGTCACC	60	10	80
<b>iNOS</b>	AGCTCCTCCC AGGACCACAC	ACGCTGAGTA CCTCATTTGGC	60	19	85
<b>rs12</b>	GGAAGGCATA GCTGCTGGAG GT	CGATGACATC CTTGGCCTGA	60	10	80

### **Histology and immunohistochemistry**

Lungs were fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections were stained with haemotoxylin and eosin (H&E), periodic acid-Schiff (PAS) for mucus, Sirius red for collagen, Martius scarlet blue (MSB) for fibrin and Gordon and Sweets for reticulin (reticulin silver staining kit). Image analysis was performed on NIS Elements (Nikon Instruments, Tokyo, Japan). Lung sections from individual mice were assessed, and data from 3 experiments were pooled.

### **Antibody ELISAs**

Blood samples taken by tail vein bleeding or directly from mice after sacrificing were collected in plasma separator tubes (Microtainer™ SST, BD, USA). Samples were centrifuged for 20min at 6000rcf and stored at -80°C. OVA specific antibodies were measured in blood serum by endpoint titration ELISA. For OVA specific antibodies, 96 well ELISA plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl OVA in PBS (5µg/ml for IgG1, IgG2a, IgG2b or 1mg/ml for IgE). For total IgE ELISA, plates were coated with anti-mouse IgE antibody (clone 84.1C, 1/1000 dilution). Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Serial dilutions of serum samples in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated overnight at 4 °C. Purified recombinant mouse IgE (BD, USA) was used as a standard for total IgE ELISA, at a starting concentration of 1µg/ml. 50µl alkaline phosphatase conjugated goat anti-mouse isotype specific antibodies (Southern Biotechnology, USA) at a 1/1000 dilution in PBS/ 0.1% BSA were added to the wells and the plates were incubated for 3h at 37°C. Subsequently, plates were incubated with 50µl pnitrophenylphosphate (Fluka, Switzerland) and colour reaction was stopped with 50µl 1M NaOH. Absorption was measured at 405nm with 492nm as reference wave length using a VersaMax plate reader (Molecular devices, USA). Plates were washed 4x with 200µl per well after each incubation.

### **Statistical analysis**

P values were calculated in GraphPad Prism 4 (GraphPad Software, Inc, San Diego, Calif) by using ANOVA with Bonferroni's posttest or repeated measures ANOVA, and results are presented as mean ± SD of the mean. Values  $p < 0.05$  was considered significant.

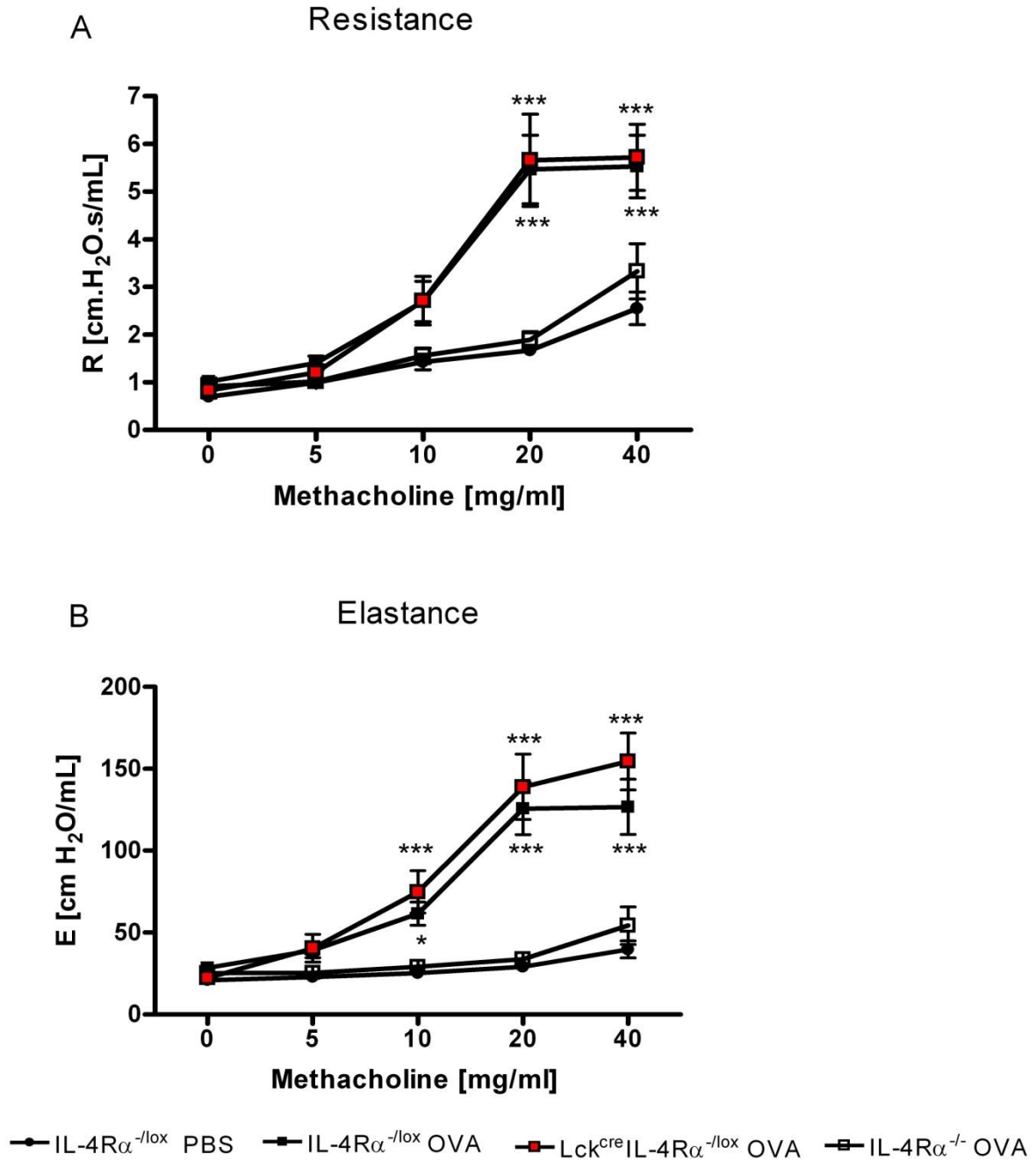
### 3.4 Results

#### 3.4.1. $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$ mice are not protected against the development of AHR

The airway responses of  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice and littermate controls were examined in OVA-induced allergic airway disease. Airway resistance (R) and elastance (E) were measured in response to increasing doses of methacholine after chronic exposure to OVA. Airway dynamic resistance (R) quantitatively assesses the level of constriction in the lungs. Increased R values signal constriction of the lungs and this parameter reflects not only central airway resistance, but is also influenced by the lung periphery (the "tissues"). Dynamic elastance (E) captures the elastic rigidity of the lungs and reflects the mechanical properties of the lungs for normal respiratory rates. Increased E values signal an increased stiffness of the lungs.

The OVA sensitised and challenged mice developed AHR, with significant differences between  $\text{IL-4R}\alpha^{-/\text{lox}}$  PBS mice littermate controls and both  $\text{IL-4R}\alpha^{-/\text{lox}}$  and  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice (Fig.1 A, B). A trend toward a slight, but not significant, increase in both airway resistance and elastance was observed in  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice compared to  $\text{IL-4R}\alpha^{-/\text{lox}}$  littermate controls. Global  $\text{IL-4R}\alpha^{-/-}$  mice did not show a significant increase in AHR compared with saline controls, demonstrating protection. Since airway resistance assesses constriction of the airways, an increase in E values may indicate similar airway remodeling in  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice and littermate controls. These results provide strong evidence that the absence of IL-4R $\alpha$  dependent AAMs does not protect against AHR.

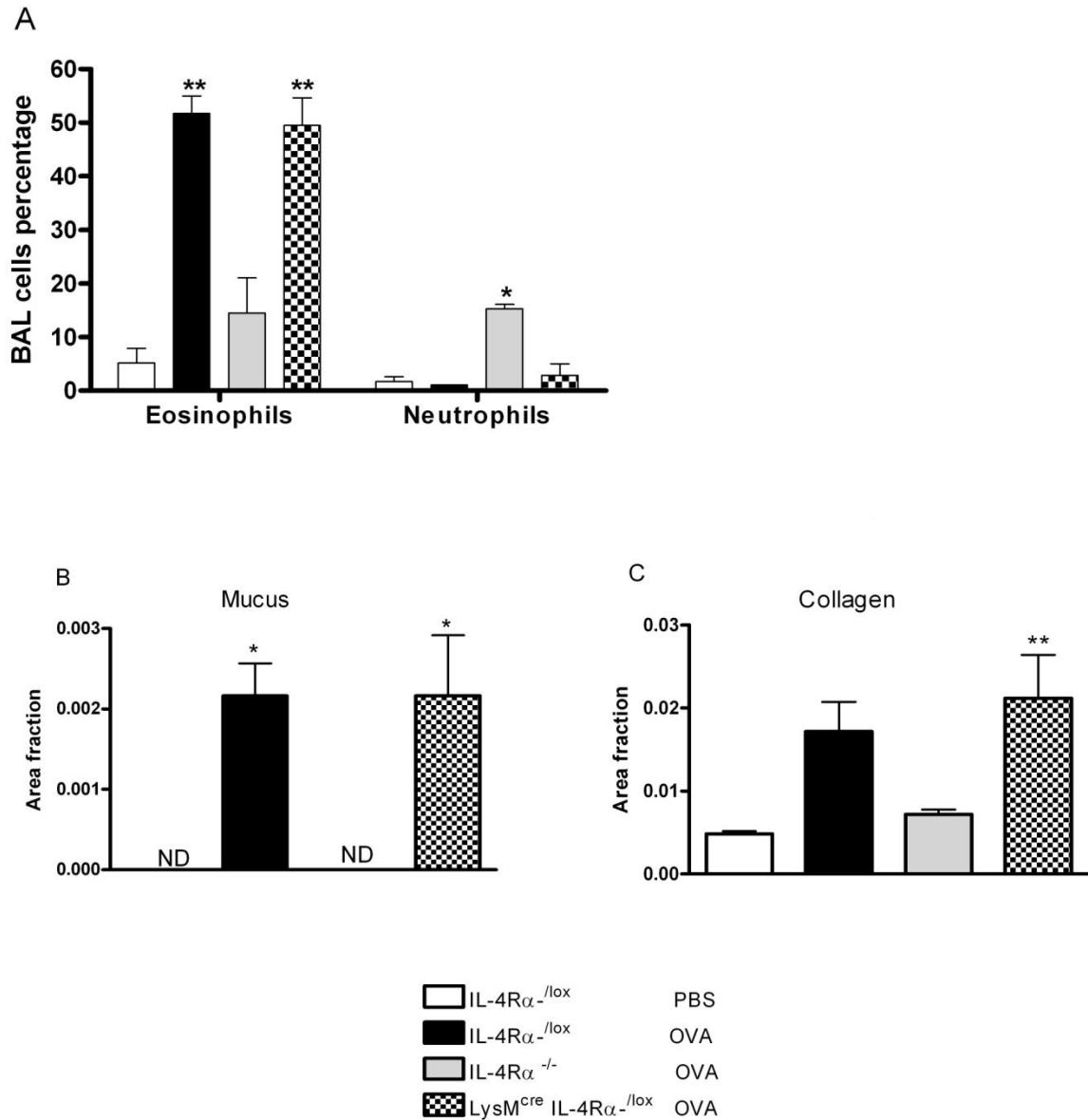
## Airway Hyper Responsiveness (AHR)



**Figure 1. LysM<sup>cre</sup>IL-4R $\alpha^{-/-}$  OVA mice are not protected against AHR.** Airway Resistance (A) and elastance (B) were significantly increased in LysM<sup>cre</sup>IL-4R $\alpha^{-/-}$  OVA mice compared to IL-4R $\alpha^{-/-}$  PBS mice. N= 10-16 mice, pooled data from three individual experiments. Significant differences between IL-4R $\alpha^{-/-}$  PBS and LysM<sup>cre</sup> IL-4R $\alpha^{-/-}$  mice are represented by stars (\*p<0.01, \*\*\*, P<0.001). P values are in comparison to individual responses and different concentrations of inhaled methacholine.

### **3.4.2. Markers of allergic inflammation (Airway infiltration, mucus production and collagen deposition) are maintained in $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$ mice**

During AAD, as a consequence of OVA sensitisation and challenge inflammatory cells were recruited to the lungs around airways and blood vessels. Airway infiltration of the lung tissue with inflammatory cells, goblet cell hyperplasia and collagen deposition was not affected by loss of IL-4 $\alpha$  on macrophages and neutrophils (Figs 2, 3). Analysis of the cellular composition of BAL fluid showed that IL-4R $\alpha^{-/\text{lox}}$  and  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice sensitised and challenged with OVA both had significantly increased percentages of eosinophils compared with PBS controls (Fig 2A). IL-4R $\alpha^{-/-}$  OVA mice showed significant increase in percentage of neutrophils compared to PBS littermate controls. IL-4R $\alpha^{-/\text{lox}}$  and  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice sensitised and challenged with OVA showed decreased percentages of neutrophils similar to PBS controls (Fig 2A). IL-4R $\alpha^{-/\text{lox}}$  OVA and  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  OVA mice had increased mucus hypersecretion (quantified by image Analysis on NIS Elements software) compared with PBS controls but abrogated in the global IL-4R $\alpha^{-/-}$  strain (Fig 2B).

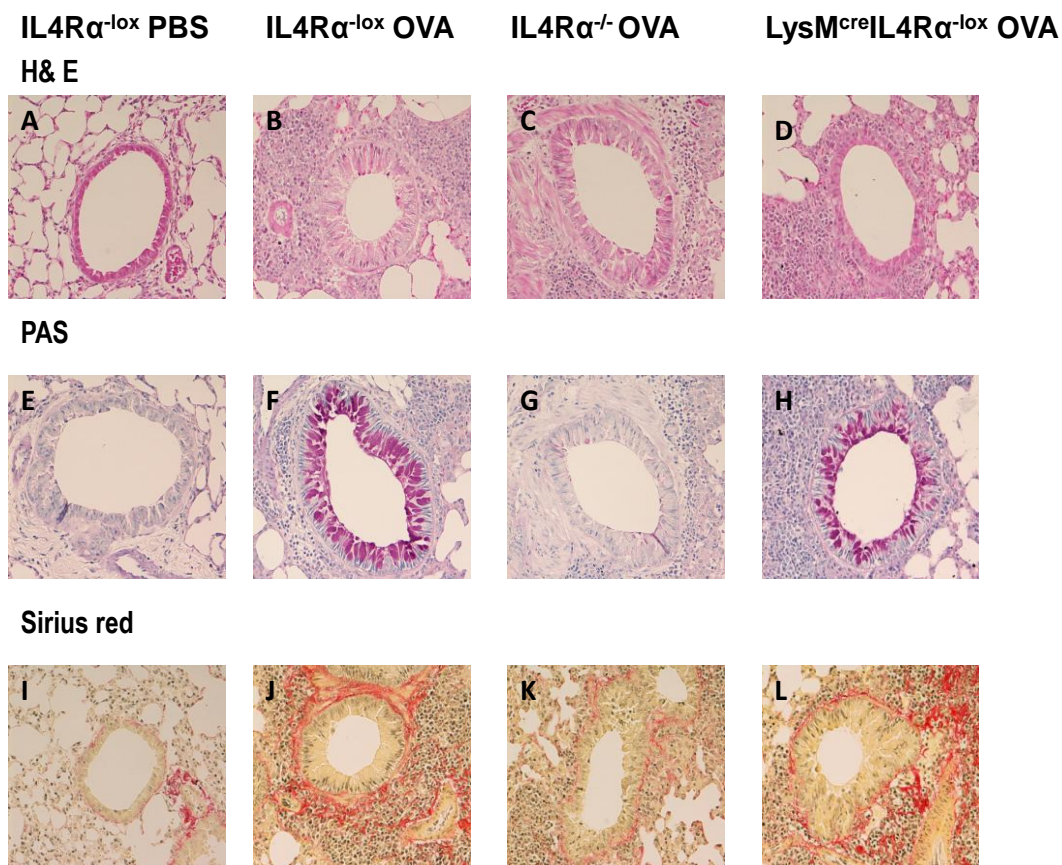


**Figure 2. Markers of lung inflammation are affected in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> OVA mice.** BAL fluid (A) showed significant increase of eosinophils in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice sensitised and challenged with OVA compared to IL-4R $\alpha$ <sup>-/-</sup> mice sensitised and challenged with saline. Mucus hypersecretion (B) and collagen deposition (C) was similar in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice sensitised and challenged with OVA mice but abrogated in the global IL-4R $\alpha$ <sup>-/-</sup> mice. N= 8-12 mice, pooled data from two different experiments. Significance was to IL-4R $\alpha$ <sup>-/-</sup> PBS. (n=6, \*, P<0.05, \*\* P<0.01)



H&E staining showed infiltration of inflammatory cells around the airways in IL-4R $\alpha$ <sup>-lox</sup> and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (Fig. 3B, D). Mucus hypersecretion by goblet cells is a characteristic of AAD. Histological examination of lungs stained with PAS showed no signs of mucus hypersecretion in the airways of PBS sensitised and challenged IL-4R $\alpha$ <sup>-lox</sup> mice (Fig. 3E) and IL-4R $\alpha$ <sup>-/-</sup> OVA mice (Fig. 3G). OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-lox</sup> mice (Fig. 3F), and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (Fig. 3H) revealed mucus hyper secretion showing similar severity of goblet cell hyperplasia. Sirius red staining showed increased collagen deposition around airways and blood vessels in IL-4R $\alpha$ <sup>-lox</sup> OVA (Fig. 3J) and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice (Fig. 3L) compared to the mice sensitised and challenged with saline /PBS (Fig. 3I) and OVA sensitised and challenged IL-4R $\alpha$ <sup>-/-</sup> mice (Fig. 3K) .

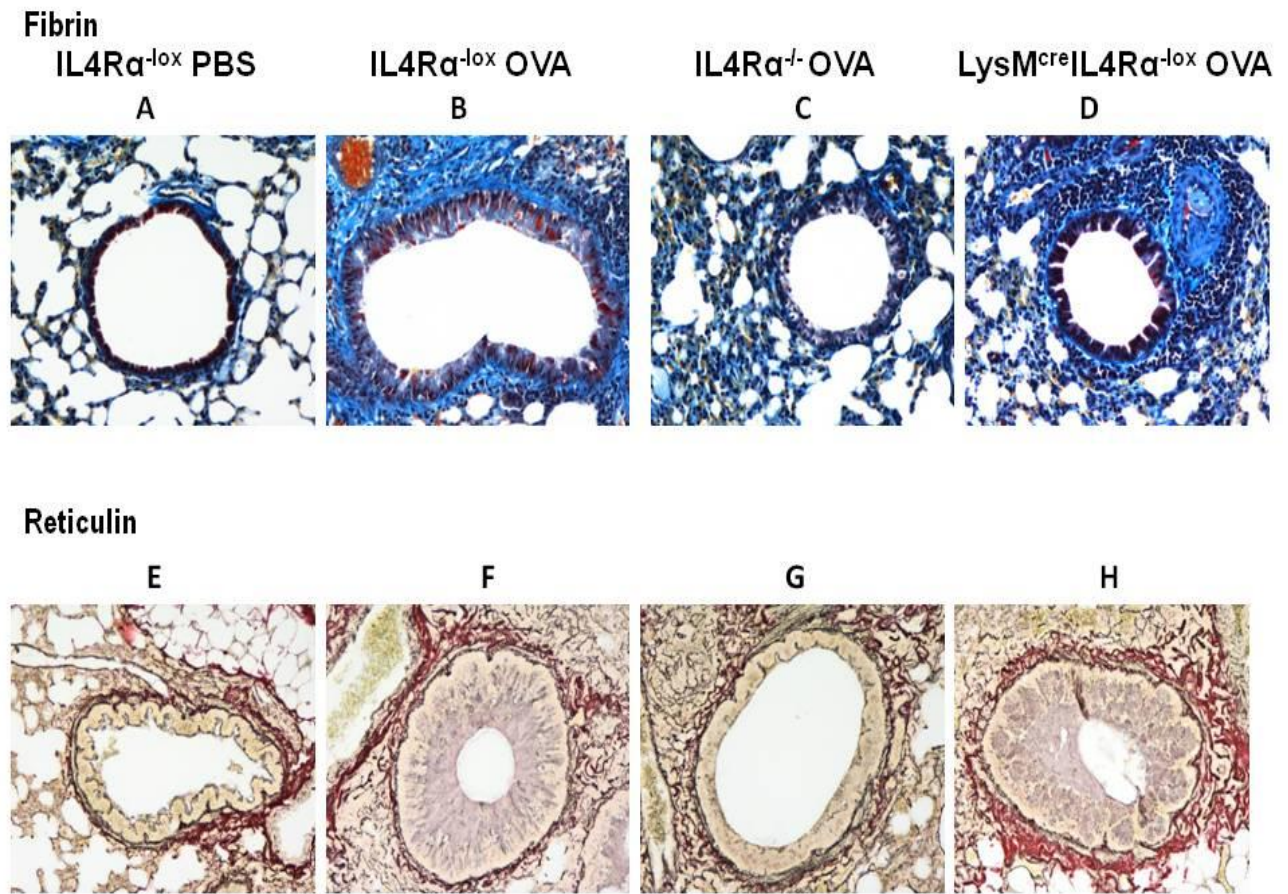
Increased levels of collagen deposition were observed in IL-4R $\alpha$ <sup>-lox</sup> OVA and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> OVA mice compared to IL-4R $\alpha$ <sup>-lox</sup> PBS and IL-4R $\alpha$ <sup>-/-</sup> OVA mice. Literature suggested that AAMs may play an important role in collagen deposition through increased production of prolines. However our data illustrate that IL-4R $\alpha$  dependent AAMs are not necessary for the development of OVA-induced allergic airway inflammation and do not appear to regulate collagen deposition.



**Figure 3. Airway infiltration of the lung tissue with inflammatory cells, goblet cell hyperplasia and collagen deposition is not affected by loss of IL-4α on macrophages and neutrophils.** Lung sections from IL-4Rα<sup>-lox</sup> mice sensitised and challenged with saline /PBS (A) and OVA sensitised and challenged littermate control IL-4Rα<sup>-lox</sup> mice (B), IL-4Rα<sup>-/-</sup> mice (C) and LysM<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice (D) were stained with H&E. Mucus hypersecretion by goblet cells were stained with PAS in the airways of PBS sensitised and challenged IL-4Rα<sup>-lox</sup> mice (E) and OVA sensitised and challenged littermate control IL-4Rα<sup>-lox</sup> mice (F), IL-4Rα<sup>-/-</sup> mice (G) and LysM<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice (H). Collagen deposition around airways and blood vessels were illustrated by Sirius red staining in IL-4Rα<sup>-lox</sup> mice sensitised and challenged with saline /PBS (I) and OVA sensitised and challenged littermate control IL-4Rα<sup>-lox</sup> mice (J), IL-4Rα<sup>-/-</sup> mice (K) and LysM<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice (L).

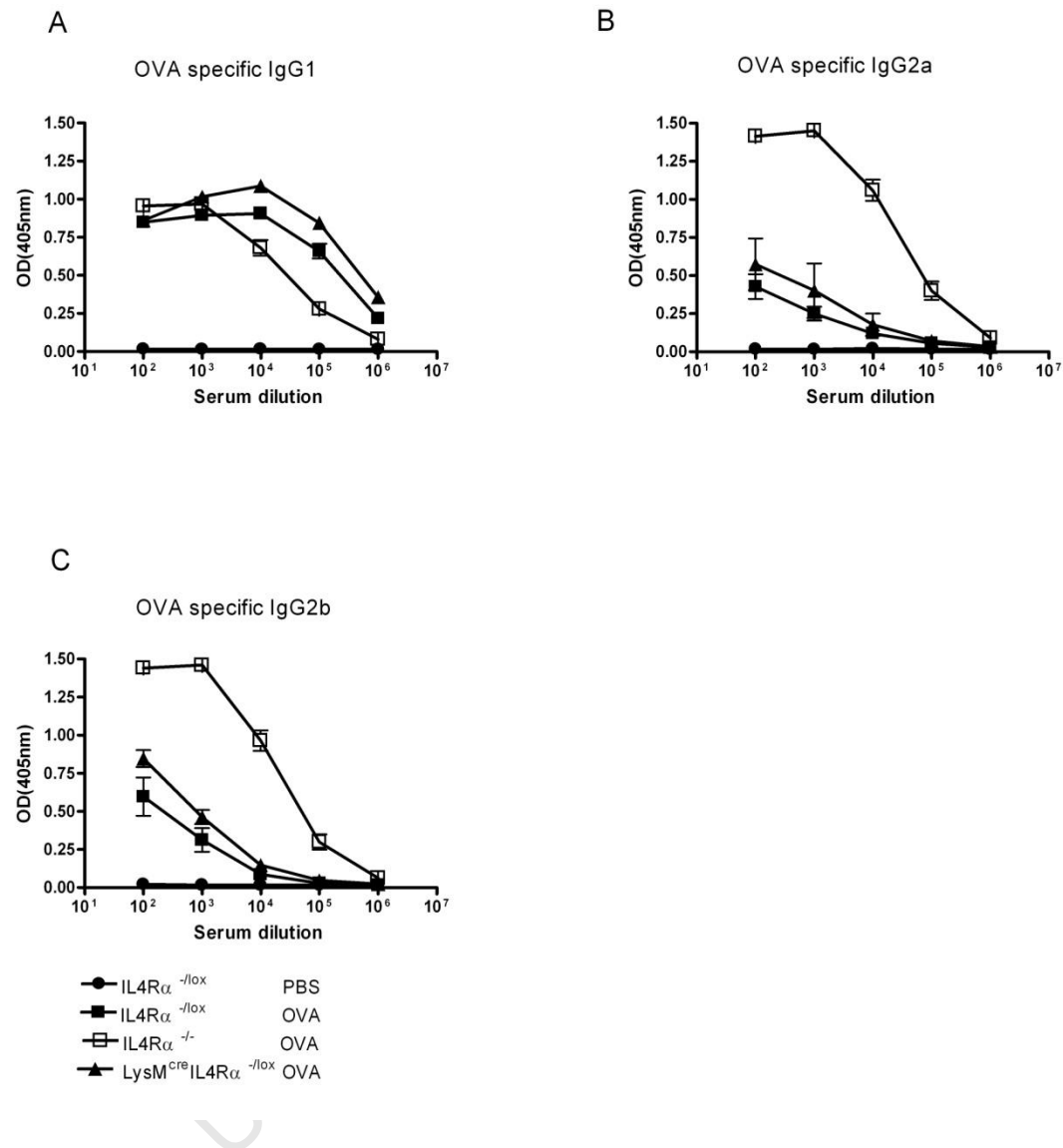
Fibrin deposition occurs as a result of inflammatory reactions in AAD. Martius Scarlet blue staining showed increased fibrin mass around the airways of OVA sensitised and challenged IL-4R $\alpha$ <sup>-/-lox</sup> littermate control mice (Fig. 4B) compared to IL-4R $\alpha$ <sup>-/-lox</sup> PBS mice (Fig. 4A). IL-4R $\alpha$ <sup>-/-</sup> OVA mice showed no fibrin threads (Fig. 4C). However LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> OVA mice revealed increased fibrin mass around the airways (Fig. 4D) similar to IL-4R $\alpha$ <sup>-/-lox</sup> OVA mice.

In chronic AAD, airway remodelling includes structural changes such as epithelial shedding and increased subepithelial collagen deposition. Reticulin silver staining showed increased thickness of basement membrane and loose arrays of reticulin fibres in the airways of OVA sensitised and challenged IL-4R $\alpha$ <sup>-/-lox</sup> littermate control mice (Fig. 4F) and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> OVA mice (Fig. 4H). Control IL-4R $\alpha$ <sup>-/-lox</sup> PBS littermate control mice and IL-4R $\alpha$ <sup>-/-</sup> OVA mice showed thick array of reticulin fibres showing the absence of airway remodelling (Fig. 4E and 4G).



**Figure 4. Airway remodelling is not affected by the loss of IL-4R $\alpha$  in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> mice.** Lung sections from IL-4R $\alpha$ <sup>-/-</sup> mice sensitised and challenged with saline /PBS (A) and OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-/-</sup> mice (B), IL-4R $\alpha$ <sup>-/-</sup> mice (C) and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> mice (D) were stained with Martius scarlet blue. Increased fibrin threads (stained red) were observed in the airways of OVA sensitised and challenged IL-4R $\alpha$ <sup>-/-</sup> mice (B) and in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> mice (D). Epithelial thickness and subepithelial collagen deposition were illustrated by reticulin fibre staining. OVA sensitised and challenged littermate control IL-4R $\alpha$ <sup>-/-</sup> mice (F) and LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-</sup> mice (H) showed loose arrays of reticular fibres (stained black) around the alveolar walls and increased collagen deposition (in red) around the airways compared to littermate control PBS mice (E) and IL-4R $\alpha$ <sup>-/-</sup> mice (G).

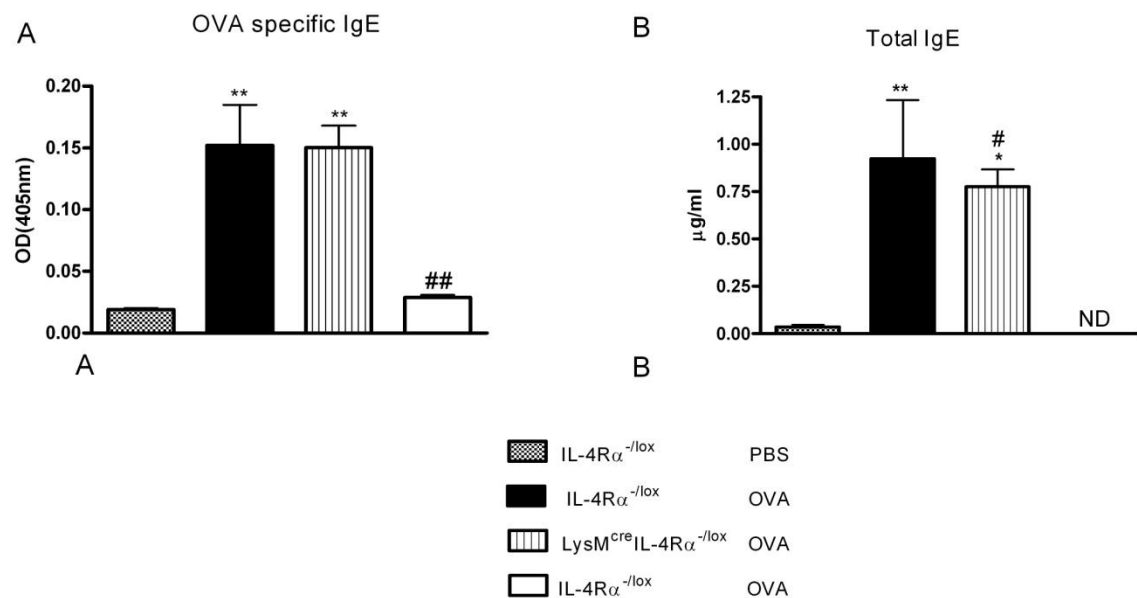
### 3.4.3. Th2 type antibody, Th2 cytokines and Th17 responses are unaltered in $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$ mice



**Figure 5.** OVA specific antibody response of  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  is similar to  $\text{IL-4R}\alpha^{-/\text{lox}}$  littermate control mice and is predominantly a Th2-type response. OVA specific antibodies were measured by end point titration ELISA. IgG1 (A), IgG2a (B), IgG2b (C) subclasses were tested. Data represents three individual experiments (N=5 mice).

In order to investigate if the antibody responses to chronic OVA sensitisation and challenge are affected by IL-4R $\alpha$  signalling on macrophages/neutrophils, OVA specific antibody levels in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were compared to those of IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice. OVA-specific IgG levels were similar in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/lox</sup> compared to IL-4R $\alpha$ <sup>-/lox</sup> PBS and IL-4R $\alpha$ <sup>-/-</sup> mice. A higher IgG1 but lower IgG2a and IgG2b titers in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice compared to IL-4R $\alpha$ <sup>-/-</sup> mice indicated a predominant Th2-type response. (Fig 5 A, B,C).

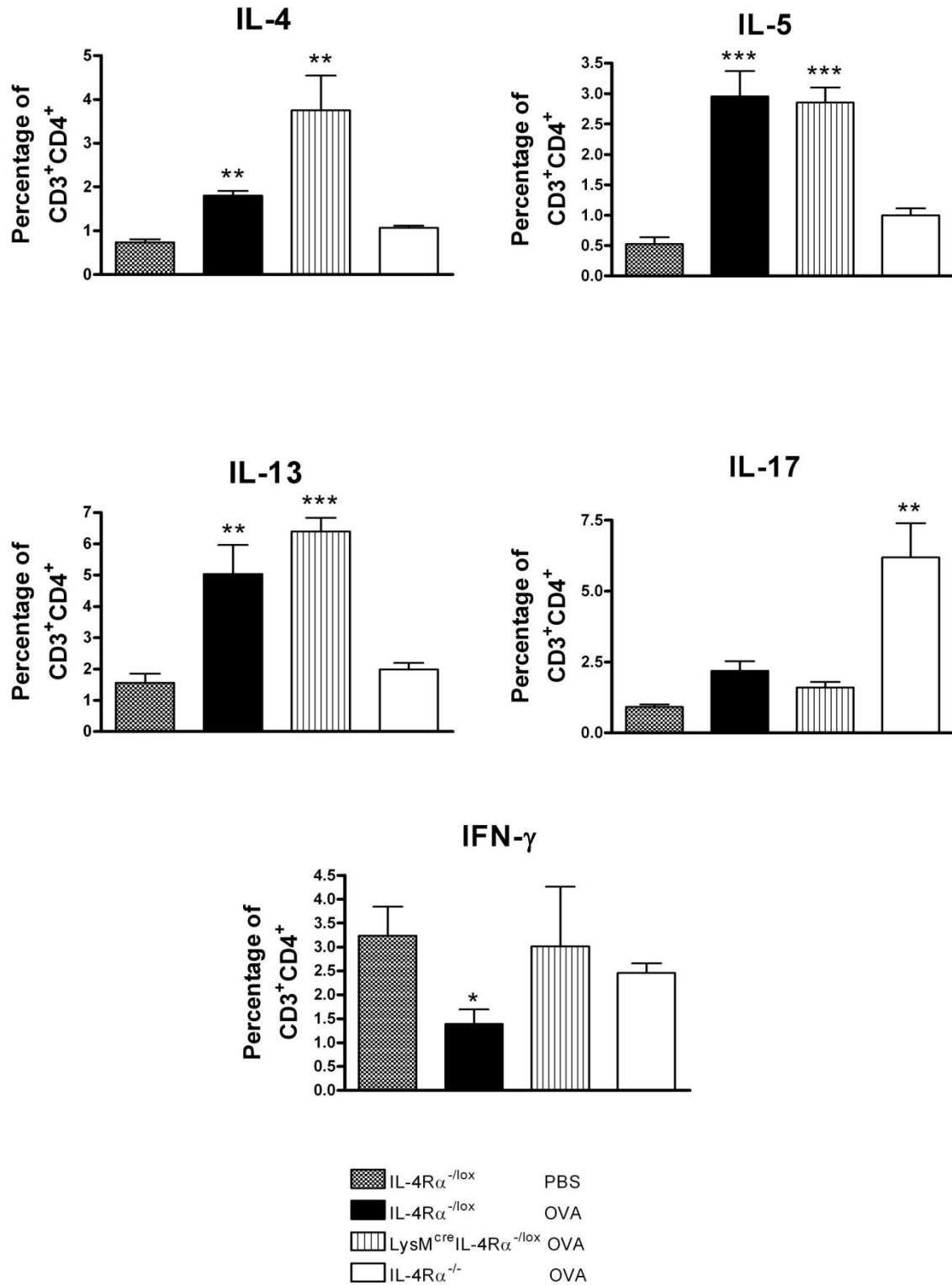
Ovalbumin specific IgE (Fig 6A) and total IgE concentrations (Fig 6B) were similar in both LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/lox</sup> mice sensitised and challenged with OVA compared to those of IL-4R $\alpha$ <sup>-/lox</sup> PBS and IL-4R $\alpha$ <sup>-/-</sup> mice.



**Figure 6. OVA specific and Total IgE antibody levels of LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/lox</sup> littermate control mice showed similar increase.** OVA specific IgE (A) and total IgE (B) antibodies were measured by end point titration ELISA. Significant differences to IL-4R $\alpha$ <sup>-/lox</sup> PBS mice (\*, p<0.05, \*\* p<0.01), Significant differences to IL-4R $\alpha$ <sup>-/lox</sup> OVA mice (#, P<0.05, ## P<0.01, ND Non Detectable).

The percentages of IL-4, IL-5 and IL-13 producing CD4<sup>+</sup> T cells was significantly increased in the lungs of IL-4Rα<sup>-lox</sup> OVA and LysM<sup>cre</sup>IL-4Rα<sup>-lox</sup> OVA mice compared with PBS littermate controls. The percentage of IFNγ producing CD4<sup>+</sup> T cells was significantly decreased in the lungs of IL-4Rα<sup>-lox</sup> OVA mice compared with PBS littermate controls. Only IL-4 was increased in LysM<sup>cre</sup>IL-4Rα<sup>-lox</sup> OVA mice compared to OVA littermate control mice. IL-17 production was significantly increased only in IL-4Rα<sup>-/-</sup> OVA mice. (Fig 7) Therefore, the absence of IL-4Rα dependent AAMs did not reduce Th2 responses or affect Th17 responses in allergic airway disease.

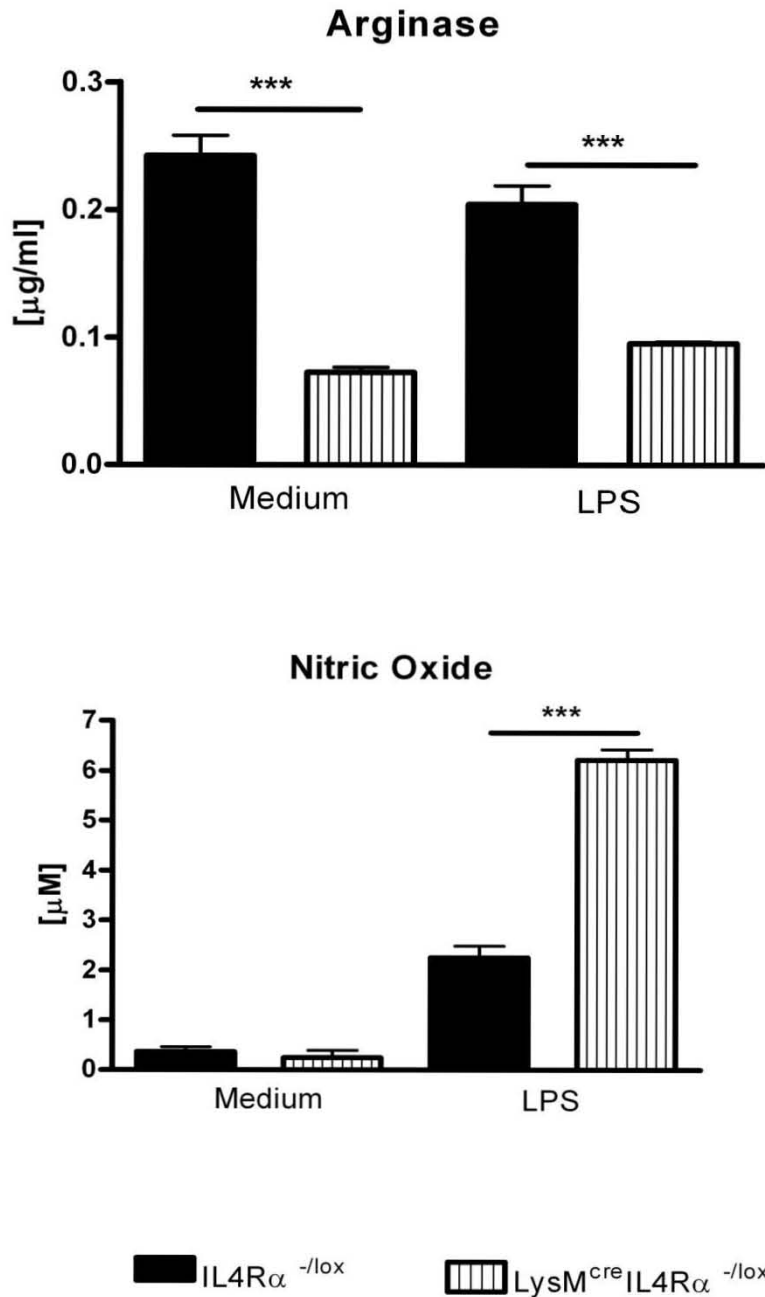
University of Cape Town



**Figure 7. Sensitisation and challenge with OVA induces a Th2 response in IL-4Rα<sup>-/-</sup> and LysM<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice.** Lung cells were incubated at 37°C for 4 hours with phorbol myristate acetate (PMA), ionomycin and monensin for intracellular cytokine staining. Significant differences to IL-4Rα<sup>-/-</sup> mice sensitised and challenged with PBS. N=5-8 mice, pooled data from two individual experiments. Significant differences to IL-4Rα<sup>-/-</sup> mice sensitised and challenged with PBS. (\*\*, P<0.01, \*\*\* P<0.001).

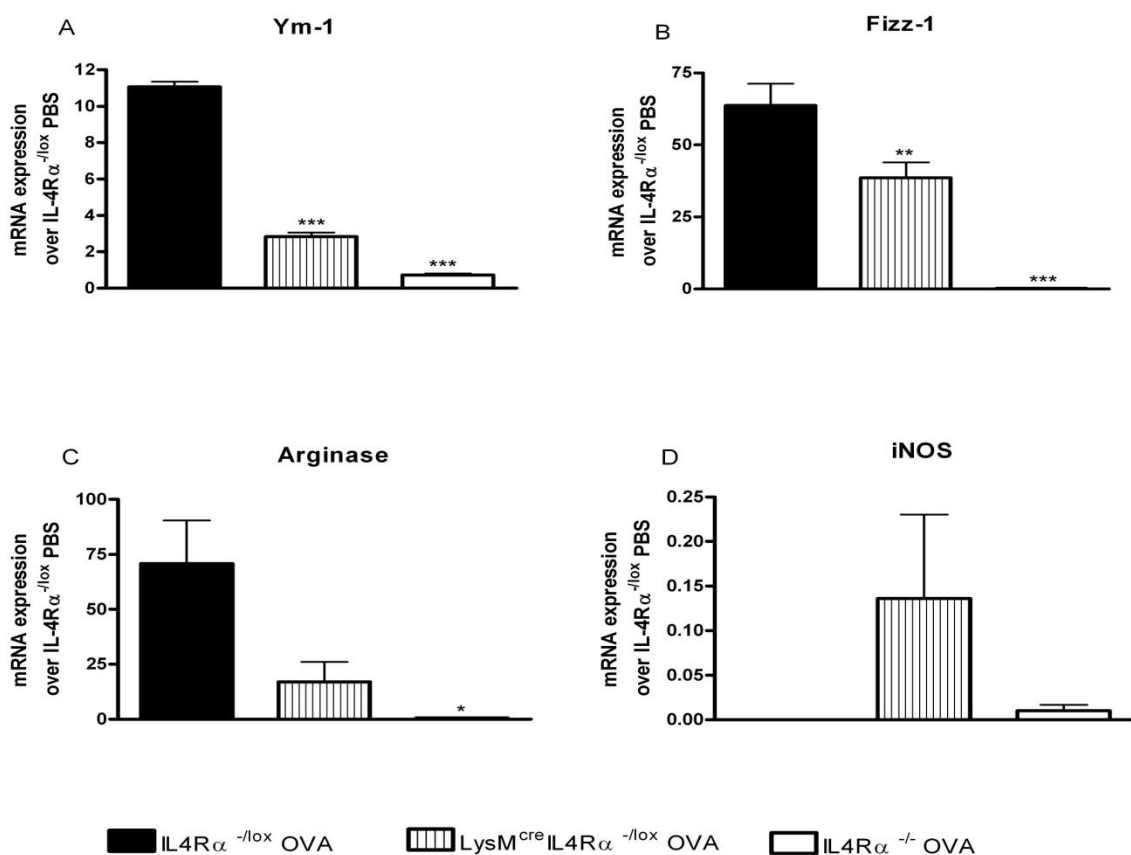


*Ex vivo* assays were performed on alveolar macrophages of  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  OVA as described earlier. There was a significant reduction of arginase activity and significant increase in iNOS activity when compared to  $\text{IL-4R}\alpha^{-/\text{lox}}$  mice. (Fig.8)



**Figure 8. Reduced arginase activity and increased Nitric Oxide (NO) production.** Alveolar macrophages of  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice showed significant decrease in arginase activity and significant increase in iNos activity compared to littermate control. (\*\*\* $P < 0.001$ ).

Alveolar macrophages from OVA sensitised and challenged mice were sorted. mRNA expression of markers for classical activation (iNOS) and alternative activation (Ym-1, FIZZ-1, and Arg-1) was quantified using RT-PCR using rs12 housekeeping gene for normalising. IL-4R $\alpha$ <sup>-/-lox</sup> mice showed alternative activation of macrophages as there was increased expression of Ym-1, FIZZ-1, and arginase in comparison to saline controls. (Fig. 8 A, B, C). The expression of arginase, Ym-1, and FIZZ-1 was decreased in macrophages of LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice compared to IL-4R $\alpha$ <sup>-/-lox</sup> mice and. iNOS expression was increased showing that in the absence of IL-4R $\alpha$  signaling there is reduced alternative activation of macrophages.



**Figure 9. mRNA expression in alveolar macrophages normalized with rs12 housekeeping gene.** Expression of Ym-1(A), Fizz-1 (B) and arginase were reduced and iNOS expression was increased in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-/-lox</sup> mice compared to IL-4R $\alpha$ <sup>-/-lox</sup> PBS mice. Data is representative of 2 experiments with triplicate pooled samples.

### 3.5 Discussion

The pathology of allergic asthma involving the contribution of cytokines such as IL-4 and IL-13 and its receptor IL-4R $\alpha$  is well understood.<sup>63-66</sup> The role of AAMs in Th2 driven pathologies such as nematode infections<sup>32</sup> and parasitic infections<sup>67</sup> has been reported. Ovalbumin induced mouse model have also revealed the role of AAMs in the aggravation of allergic airway inflammation.<sup>68</sup> However, the role of AAMs in AAD remains unclear. To date, there is no clear evidence that exists to indicate that AAMs directly play a role in AAD although an upregulation of AAM markers in murine AAD and human asthma has been shown.<sup>1,9,74</sup> Previous studies from our group have demonstrated the expression of AAM markers that are IL-4R $\alpha$  independent.<sup>69,70</sup> Other parasitic model studies such as *Schistosoma mansoni* conducted by our co-workers have proven the suppression of fibrosis by AAMs expressing certain molecules.<sup>69</sup> Human and mice studies have demonstrated, enhanced eosinophilic inflammation in the presence of AAMs.<sup>20, 71-83</sup>

There has been evidence to suggest that that immunological overreaction in the lungs is prevented by macrophages.<sup>55, 84-86</sup> either by suppressing T cell proliferation and activation after exposure to inhaled antigens. An exacerbation of AHR, eosinophil inflammation and Th2 cytokine secretion due to depletion of alveolar macrophages has also been reported.<sup>87</sup> It is known that interstitial macrophages suppress AAD through the production of IL-10.<sup>5</sup> AAMs downmodulate inflammation in several disease models<sup>24, 37, 39</sup> and their role in the suppression of T-cell proliferation has been demonstrated.<sup>6, 15, 26, 37, 42, 43, 88</sup> In helminth models, it has been demonstrated that FIZZ-1 (a marker of AAM) regulates Th2 responses and associated inflammation negatively.<sup>89</sup>

A previous study from our group has demonstrated that the presence of AAMs is not required for the onset of AAD in an acute model.<sup>90</sup> In this study, we investigated the cell specific contribution of IL-4R $\alpha$  dependent AAMs in a chronic model of murine AAD. LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice which have impaired IL-4R $\alpha$  signalling in macrophages and neutrophils and therefore lack IL-4/IL-13-induced activation of macrophages through IL-4 receptor types 1 and 2 were sensitised and challenged with ovalbumin. In comparison to IL-4R $\alpha$ <sup>-lox</sup> mice sensitised and challenged with PBS. We observed a significant and consistent increase in airway resistance and elastance in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice similar to that of OVA littermate controls (IL-4R $\alpha$ <sup>-lox</sup>) after methacholine challenge. AHR, airway inflammation, mucus

hypersecretion, and collagen deposition in  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice were similar to littermate controls in spite of impaired activation of AAMs. This observation suggests that IL-4R $\alpha$ -dependent AAMs do not play an important role in the regulation of collagen deposition, as suggested on the basis of their expression of arginases. Hence, demonstrating that IL-4R $\alpha$ -dependent AAMs are not necessary in the pathogenesis of murine AAD. It is known that arginases compete with nitric oxide synthases (NOS) for the substrate arginine. While NOS uses arginine to produce bacteriocidal NO, arginases convert arginine into ornithine and urea, with downstream products that include proline and polyamines, used in collagen synthesis and cell growth, respectively.<sup>91</sup>

Mimicking previous studies,<sup>2, 27, 28, 82, 91-94</sup> there is an upregulation of AAM markers such as arginase, Ym-1, and Fizz-1 in OVA littermate control mice ( $\text{IL-4R}\alpha^{-/\text{lox}}$ ) mice during OVA-induced allergic airway disease. We observed an impaired expression of these markers on alveolar macrophages in  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice, although they still exhibited a slight increase compared to the PBS littermate control mice ( $\text{IL-4R}\alpha^{-/\text{lox}}$ ). Based on the results obtained, it can be deduced that impaired expression of Ym-1 in the  $\text{LysM}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$  mice suggests that AAMs are not involved in the recruitment of eosinophils. It has been established in murine studies that AAM marker does not contribute to AAD<sup>96</sup> which corresponds to our findings. Hence it is also vital to understand the mechanism by which alternative activation influences the phenotype of distinct macrophage populations. However, other murine studies have shown that adoptive transfer of IL-4R $\alpha^{+/+}$  M-CSF-dependent macrophages in the presence of Th2 cells, significantly contributed to OVA-induced recruitment of eosinophils to the lung.<sup>97</sup> In addition, stimulation of macrophages through IL-4R $\alpha$  result in their alternative activation enhancing the Th2-driven allergic inflammatory response in the lung.<sup>97</sup>

In conclusion, our results suggest that association between alternatively activated macrophages and allergic airway disease does not translate into a pathological role for these cells in AAD. This suggests that IL-4R $\alpha$  activated AAMs are merely bystander cells responding to the Th2 cytokines. They do not play a significant role in the pathology of AAD and are not detrimental in murine AAD. Airway hyperreactivity was increased in the absence of AAMs, suggesting that the presence of alternative activation of macrophages in AADs may be an attempt to downregulate excessive immune effector responses. Despite the importance of global IL-4R $\alpha$  expression, this also suggests that effector functions of other

cell types are essential in the development of AHR and pathology of AAD. Thus AAMs in allergic airway disease may be merely associative but not causative and is a result of elevated Th2 responses.

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### 3.6 References

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## **CONCLUSIONS**

## Conclusions

The aim of this study is to investigate the cell specific contributions of IL-4R $\alpha$  signalling on the outcome of Ovalbumin-induced chronic allergic airway disease (AAD) in mice. Specifically we aimed to investigate the role of IL-4/IL-13 responsive CD4<sup>+</sup> T cells in AAD, using Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice and the role of IL-4/IL-13 induced alternatively activated macrophages (AAM) in AAD, using LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice.

Allergic asthma shows Th2 inflammatory responses in the lung orchestrated by CD4<sup>+</sup> T cells. Most murine models and human studies have shown that CD4<sup>+</sup> T cells infiltration around the airways and eosinophil recruitment are key events of the inflammatory process contributing to disease severity. The direct role of IL-4R $\alpha$  on CD4<sup>+</sup> T cells in chronic allergic airway disease and airway remodelling is yet to be explored. Previously characterized and published Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice were sensitized and challenged with ovalbumin.

Our data in the present study shows Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice to be significantly protected from AAD in comparison to OVA IL-4R $\alpha$ <sup>-lox</sup> littermate control mice showing a significant decrease in AHR, airway inflammation, mucus hypersecretion, collagen deposition and airway remodelling. Although it has been characterised that CD4<sup>+</sup> T cells are required at later stages in establishing AAD, our data provides a strong clue that IL-4R $\alpha$  dependent CD4<sup>+</sup> T cells contribute significantly to the pathology of chronic AAD. The results presented here clearly demonstrate the importance of IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells. Our first major conclusion from the study is that IL-4R $\alpha$  signalling on CD4<sup>+</sup> T cells plays an essential role in murine AAD.

IL-4, IL-13 and its receptor IL-4R $\alpha$ 's role in the pathology of allergic asthma has been understood well. The contribution of AAMs to certain Th2 driven pathologies has also been studied. The role of AAMs in the aggravation of allergic airway inflammation has been confirmed in Ovalbumin-induced mouse model. However, the role of AAMs in AAD has remained unclear and no clear evidence exists yet to show that AAMs directly play a role in allergic airway disease although an upregulation of AAM markers was observed in murine AAD and human asthma.

The current study dissected the cell specific contribution of IL-4R $\alpha$  dependent AAMs in murine AAD. LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice which lack IL-4R $\alpha$  signalling in macrophages and neutrophils and therefore lack IL-4/IL-13-induced activation of macrophages through IL-4 receptor types 1 and 2 were sensitised and challenged with ovalbumin. In comparison to IL-4R $\alpha$ <sup>-lox</sup> mice sensitised and challenged with PBS, we found a significant and consistent increase in airway resistance and elastance in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice after methacholine challenge similar to littermate controls. AHR, airway inflammation, mucus hypersecretion, collagen deposition and airway remodelling in LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice were similar to littermate controls inspite of impaired activation of AAMs suggesting that IL-4/IL-13 induced IL-4R $\alpha$ -dependent alternatively activated macrophages (AAM) do not play an important role in the pathogenesis of chronic murine AAD.

Our second main conclusion highlighted the association between alternatively activated macrophages and AAD which does not translate into a pathological role in AAD suggesting that IL-4R $\alpha$  activated AAMs do not play a key role in the pathology of AAD. This provides a strong evidence that AAMs in allergic airway disease may be merely associative but not causative and is a result of elevated Th2 responses.